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**University of Alberta**

# The Role of Alternative Multiubiquitin Chains in Ubiquitin-dependent Processes.

by

**Terra Gayle Arnason**

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

Fall, 1995

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Dated: Oct. 2, 1995

To my husband Troy for making the achievement all that much more worthwhile.

**Abstract**

The covalent attachment of ubiquitin (Ub) to short lived or damaged proteins through a branched, or isopeptide, bond can function as a signal that initiates their selective degradation. The degradative signal can take the form of a multiUb chain in which successive Ub molecules are linked tandemly through lysine 48 (K48). Ub-Ub conjugates linked through residues other than K48 have been reported, yet their linkage position and biological functions have not been determined. The work in this thesis has identified the alternative sites of Ub conjugation *in vivo* in *Saccharomyces cerevisiae* at positions K29 and K63. Furthermore, of the two novel linkages, neither is apparently involved in bulk protein degradation. Additionally, the K63 conjugate is demonstrated to be involved in stress resistance.

The formation of the alternative linkages is strongly dependent on the presence of the Ub-conjugating enzymes (E2s) RAD6, UBC1, in addition to UBC4 and its homolog UBC5 which, like the K63 Ub linkage, are necessary for stress resistance. Functional analysis of UBC4 derivatives reveal that three surface exposed amino acid residues are involved in UBC4 stress resistance, and also suggested that different stresses may involved different cellular pathways. It is therefore proposed that these UBC4 residues comprise critical contact points for associated protein factors necessary for target protein recognition, factors known to exist but which have not been identified to date. An overexpression suppression strategy was carried out to yield the REP1 transcriptional repressor protein, encoded by the endogenous yeast 2 micron plasmid, as a candidate protein factor which effects UBC4 function by physical association.

Lastly, these results imply that a greater level of regulation of the Ub system may be necessary for different E2 function than previously anticipated, as it appear that both target specificities and linkage specificities exist. Furthermore, it may be that the structural variation predicted to be part of the different Ub conjugates can be recognized as versatile intracellular signals.

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The studies presented in this thesis would not have been possible without the input from many people. Foremost was the help from Mike Ellison who has continually amazed me by his ability to take a complex problem and dissect it into ten short words. His support and encouragement have been invaluable in my floundering search for the scientific method. Also, I want to thank all those in my lab, old and new. Thanks to Elizabeth Silver and Todd and Chantelle Gwozd for their initial work in several studies I continued. Also, Karen Robinson, Kim Ellison (both of the witches club), Doug Hogue, and Chris Ptak for the many conversations and insights into research, sober or otherwise. Lastly, I thank my husband, Troy Harkness, for his support and optimism at all times. It is to him that I give my sincere thanks for making this goal achievable.

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List of Abbreviations.

| | |
|---|---|
| A | adenine |
| Ala | alanine |
| amp | ampicillin |
| Arg | arginine |
| ARS | autonomous replication sequence |
| Asp | aspartic acid |
| Asn | asparagine |
| bisacrylamide | *N,N'*-methylenebisacrylamide |
| bp | base pair |
| BSA | Bovine Serum Albumin |
| C | cytosine |
| CEN | centromere |
| Ci | Curie |
| cir° | yeast strain lacking 2 μ plasmids |
| cpm | counts per minute |
| C-terminus | carboxy terminus |
| *CUP1* | copper metallothionine promoter |
| *CYC1* | *CYC1* transcriptional terminator |
| Δ | delta, deletion |
| D | aspartic acid |
| DIG | digoxigenin |
| dNTP | 2'-deoxynucleotide 5'-triphosphate |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| E1 | Ub activating enzyme |
| E2 | Ub conjugating enzyme |
| E3 | Ub ligase |
| ECL | chemiluminescent |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| FPLC | Fast Pressure Liquid Chromatography |
| g | gravity |
| G | guanine |
| GST | glutathione-*S*-transferase |

| | |
|---|---|
| Gly | glycine |
| IgG | immunoglobulin G |
| K | lysine |
| Kan | kanamycin |
| kb | kilobase, 1000 bases |
| kDa | kilodalton, 1000 daltons |
| kb | kilobase, 1000 bases |
| MCS | multiple cloning site |
| Met | methionine |
| µF | microFaraday |
| mg | milligram |
| µg | microgram |
| ml | milliliter |
| µl | microliter |
| mM | millimolar |
| MonoQ | quaternary anion exchange column |
| mUb | N-terminal myc tagged Ub |
| multiUb | multiubiquitin chain |
| myc | myc epitope |
| ng | nanogram |
| nm | nanometer |
| O.D. | optical density |
| ori | origin of replication |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| Pro | proline |
| psi | pounds per square inch |
| R | arginine |
| rpm | rotations per minute |
| *S. cerevisiae* | *S. cerevisiae* |
| SDS | sodium dodecylsulphate |
| Ser | serine |
| T | thymine |
| TAE | Tris; acetate, EDTA |
| TBS | Tris-buffered saline |
| Tris | Tris (hydoxymethyl) aminomethane |

| | |
|---|---|
| *TRP1* | 5' P-ribosyl-anthranilate isomerase gene |
| *ts* | temperature sensitive |
| Tyr | tyrosine |
| Ub | wild type ubiquitin protein |
| UbΔ | ubiquitin with Gly75 and Gly76 deleted |
| Ubm | ubiquitin with a C-terminal myc tag |
| *URA3* | orotidine-5'-P-decarboxylase gene |
| ORF | open reading frame |
| UV | ultraviolet light |
| WCL | whole cell lysate |

# CHAPTER 1. The Ubiquitin system in *S. cerevisiae.*

## 1.1. Introduction.

The survival of living organisms and the individual cells of which they are composed depends on regulated growth and adaptation to various environmental conditions. Essential cellular changes necessary for cell cycle progression and survival responses require the alteration of protein levels. Protein levels are regulated by the balanced forces of protein synthesis versus protein degradation. The loss of regulation of these opposing pathways leads to many cellular perturbations, including the loss of normal cell cycle progression and the ability to overcome stress conditions. A highly regulated eukaryotic protein degradation system that is intimately involved in cell growth, adaptive changes and stress resistance is the focus of this review.

One major function of intracellular protein degradation is the selective elimination of damaged and otherwise abnormal proteins. Another role is to alter the half-lives of undamaged proteins whose intracellular concentrations must vary as a function of time (reviewed by Finley, 1992; Hershko and Ciechanover, 1992; Ciechanover and Schwartz, 1994; Pines, 1994). For instance, many regulatory proteins are extremely short lived *in vivo* (Chowdary *et al.*, 1994; Chen *et al.*, 1993; Kornitzer *et el.*, 1994, Madura and Varshavsky, 1994). Many other proteins, while naturally long lived, may become metabolically unstable in response to transient intracellular responses such as phosphorylation, cell cycle progression, or ligand binding (Kong and Chock, 1994; Yee *et al.*, 1994; Hershko *et al.*, 1994; Mori *et al.*, 1993; Cenciarelli *et al.*, 1992).

There are numerous proteolytic enzymes in both prokaryotes and eukaryotes (reviewed in Jones, 1991). In eukaryotes, many of these are found in discrete intracellular compartments including the endoplasmic reticulum (Bonifacino and Lippincott-Schwartz, 1991), the lysosome or yeast vacuole (Chiang and Schekman, 1991), and mitochondria (van Dyck *et al.*, 1994; Suzuki *et al.*, 1994). In addition, eukaryotes have a major ATP-dependent cytosolic protein degradation system. Reconstitution experiments from reticulocytes revealed that a heat stable polypeptide was required for this eukaryotic degradative system (Wilkinson *et al.*, 1980). The polypeptide was subsequently identified as ubiquitin (Ub), and this proteolytic system referred to as the ATP dependent Ub-proteolytic system.

Ubiquitin is a small. noncatalytic protein, and its covalent attachment to protein substrates is required for all functions carried out by the Ub system (reviewed in Jentsch, 1992). The covalent linkage between Ub and such proteins occurs between the carboxyl

(C)-terminus of Ub and one or more ε-amino groups of lysine residues, several of which have been identified in the targeted substrate (Sokolik and Cohen, 1991; Banerjee *et al.*, 1993; Nickel and Davie, 1989; Johnson *et al.*, 1990). The resulting bond is termed an isopeptide linkage.

The selection of protein targets for ubiquitination is the role of the enzymes which form the Ub-protein linkages, and not Ub itself (reviewed in Rechsteiner, 1991; Ciechanover and Schwartz, 1994). Proteins targeted by the Ub-dependent proteolytic system include both abnormal (unfolded) and short lived (regulatory) proteins. Examples include the transcriptional regulators MATα2, (Chen *et al.*, 1993), and GCN4 (Kornitzer *et al.*, 1994), as well as cyclins (Glotzer *et al.*, 1991), transmembrane proteins such as the PDGF receptor (Mori *et al.*, 1993), the T cell receptor (Cenciarelli *et al.*, 1992 ) and the yeast uracil permease transporter (Galan *et al.*, 1994). In addition, the general class of unfolded and misfolded proteins arising from translational errors or stress conditions are also targeted for ubiquitination by the Ub proteolytic system (Parag *et al.*, 1987; Seufert and Jentsch, 1990).

The attachment of Ub to proteins to form Ub-protein conjugates has been demonstrated to result in the degradation of the targeted substrate (reviewed in Ciechanover, 1994). The recognition and proteolysis of the conjugate is carried out by the 26S proteasome, a multisubunit protein complex with several proteolytic activities. However, ubiquitination does not have to ultimately result in degradation as some ubiquitinated proteins have been shown to be stable (Paolini and Kinet, 1993). Furthermore, the attachment of Ub to protein is reversible and the Ub moiety can be precisely removed from a Ub-protein conjugate to yield both unmodified Ub and protein targets (Matsui *et al.*, 1982; Pickart and Rose, 1985). This reversible conjugation is mediated by a large class of related Ub system enzymes distinct from those involved in Ub conjugation (Xiao *et al.*, 1994; Baker *et al.*, 1992; Tobias and Varshavsky, 1991). Therefore, Ub conjugation may have other functions in addition to targeting protein for degradation, and may serve as a reversible signal analogous to phosphorylation.

Perturbations of the Ub system have revealed its involvement in diverse cellular processes. For example, it has been demonstrated that the conjugation of Ub to cellular proteins is necessary for cell cycle progression (reviewed in Pines, 1994), organelle biogenesis (Wiebel and Kunau, 1992; Crane *et al.*, 1994), stress resistance (Seufert and Jentsch, 1990, Seufert *et al.*, 1990; Jungmann *et al.*, 1993) and DNA repair (Jentsch *et al.*, 1987; Lawrence, 1994) to name a few. In most cases, neither the protein targets nor their mode of recognition has been elucidated, yet in recent years significant progress has been made towards this end. This discussion provides a current overview of the Ub system in

the yeast *Saccharomyces cerevisiae*. To be discussed are the contemporary explanations for the mechanisms of protein target selection and protein degradation, as well as focusing on the various intracellular roles and influences of multi-ubiquitination.

## 1.2. The Ub pathway

### a. The Ub genes.

Ubiquitin is encoded in *S. cerevisiae* by a family of four genes whose translational products are protein fusions (Figure 1.1). Three of the four Ub gene protein products are composed of Ub C-terminal fusions to small peptides that are components of the ribosome (Finley *et al.*, 1989). The fourth gene encodes a pentameric head-to-tail repeat of Ub with no intervening sequences (Özkaynak *et al.*, 1987). Disruption of only the Ub coding sequences within all four Ub genes is lethal, indicating that Ub is essential for cell viability (Finley *et al.*, 1994). These primary translation products are rapidly processed by Ub C-terminal proteases (also referred to as C-terminal hydrolases). This processing results in the release of free Ub monomers which are then available to be conjugated to suitable protein targets. The ribosomal subunits appended to the 3' end of the *UBI1*, *UBI2* and *UBI3* translational products are released in their native form by this processing and become incorporated into the multisubunit ribosomal complexes (Finley *et al.*, 1989).

The expression of the *UBI1*, *UBI2* and *UBI3* genes provides sufficient Ub protein for normal vegetative growth in *S cerevisiae*. Under conditions of stress, however, the polyUb gene, *UBI4*, is significantly upregulated and Ub protein levels are increased (Finley *et al.*, 1987; Fraser *et al.*, 1991; Cheng *et al.*, 1994). The *UBI4* gene contains a heat shock element (HSE) motif present in the promoter region (Finley *et al.*, 1987), allowing for *UBI4* induction under conditions of heat shock, oxidative stress, DNA damage, or abnormal protein accumulation (Ananthan *et al.*, 1986; Treger *et al.*, 1988). A deletion in the polyUb gene, *UBI4*, does not affect the growth of a yeast strain under normal conditions, but when exposed to prolonged heat stress or when grown in the presence of amino acid analogs, such strains exhibit extreme sensitivity (Finley *et al.*, 1987). Any of these stresses can cause the accumulation of abnormal proteins by thermal unfolding or amino acid analog incorporation (Kobayashi and McIntee, 1993; Menninger *et al.*, 1994). The increased demand for Ub necessary to target such proteins for subsequent degradation is likely not met in the *UBI4* deletion strain (Haas and Bright, 1985), resulting in the inability to remove these proteins and the subsequent decrease in cell viability.

**b. The Ub protein.**

Ubiquitin is a small, 8.5 kDa highly conserved 76 amino acid protein. The amino acid sequences of Ub from yeast to human have three conservative amino acid differences at positions 18, 24 and 57. The protein has 11 acidic and 11 basic residues, giving the protein an almost neutral isoelectric point of 6.7 (Wilkinson, 1988). The crystal structure of monomeric Ub reveals that the protein consists of a tightly packed globular domain, with a short flexible C-terminus extending from the core (Vijay-Kumar *et al.*, 1987). Approximately 90% of the polypeptide chain is involved in hydrogen-bonded secondary structure, perhaps accounting for the pronounced thermal stability of this protein (Wilkinson, 1988).

The C-terminus of Ub is necessary for its conjugation to cellular proteins, as demonstrated by the inability of a two amino acid Ub C-terminal deletion mutant (UbΔ) to form isopeptide bonds (Wilkinson *et al.*, 1981). Another important component of the Ub protein is the seven lysine residues exposed on the surface. Reductive methylation of the lysine residues in Ub (resulting in the modification of the ε-amino and α-amino side chains) lead to Ub-protein conjugate stabilization of a previously degraded substrate *in vitro* (reviewed in Finley, 1992) As discussed below, the function of Ub in protein degradation requires the availability of free lysine side chains.

**c. The Mechanism of Ub conjugation.**

The Ub-conjugation pathway requires the consecutive action of several classes of enzymes. The overall result is the conjugation of the C-terminal glycine residue of Ub to one or more ε-amino groups of lysines in the target protein (Figure 1.2).

The process of Ub conjugation is initiated by a specific Ub-activating enzyme (E1) in a three step reaction. The first step is the ATP-dependent activation of the C-terminus of Ub, catalyzed by E1. In this reaction, Ub first forms an adenylate intermediate before being transferred to a thiol site on E1. $Mg^{2+}$-dependent hydrolysis of ATP between the α and β phosphoryl groups drives the formation of tightly bound ubiquitin adenylate at the C-terminal Gly76 residue (Ciechanover *et al.*, 1991a; Haas *et al.*, 1982). The adenylated Ub associates with the active site sulfhydryl group of E1 (Haas *et al.*, 1982; Ciechanover *et al.*, 1982). This thiol ester linkage is formed between the C-terminus of Ub and the active site cysteine residue on E1, with the simultaneous release of AMP.

Ub is then transferred to a specific cysteine residue on one of a number of Ub-conjugating enzymes also known as E2 proteins. As is the case in E1 enzymes, this cysteine residue is the active site, and the linkage between E2 and Ub is a thio-ester bond. In the last step, E2 proteins transfer Ub to ε-amino groups of lysine residues in acceptor

proteins. It is thought that proteins with suitable structures are recognized and first bound to specific sites in E2s, with the subsequent transfer of Ub to the substrate.

Some species of E2s can transfer Ub directly to acceptor proteins. Other E2s require the presence of a Ub-ligase (E3) to catalyze the transfer of Ub to the substrate (Figure 1.3). In one instance, it has been shown that the E3 possesses an essential cysteine residue which accepts Ub from the E2-Ub intermediate before transfer to the target protein (Scheffner *et al.*, 1995). As discussed in further detail below, certain E2 enzymes appear to require the presence of distinct E3s and/or *trans*-acting factors for target recognition. Furthermore, while only a single E3 has been conclusively identified in *S. cerevisiae*, there is strong evidence to suggest that additional proteins belonging to the E3 Ub-ligase family await discovery.

Analysis of ubiquitinated substrates reveals that a number of Ub proteins can be covalently attached to a single acceptor protein. Furthermore, it was observed that more Ub moieties were present than lysine residues in the target protein. Since, in several instances it has been demonstrated that a single lysine residue in the acceptor protein is targeted for ubiquitination, these observations imply that Ub can become covalently attached to other Ub monomers in succession to form multiUb chains (Hershko and Heller, 1985; Bachmair *et al.*, 1989). The significance of these multiUb chains is fully discussed below.

**d. Ubiquitination and degradation.**

One function of the Ub system is to target proteins for selective degradation. Mutations in components of the Ub-conjugation pathway, including murine E1 and several yeast E2s, have been shown to result in the stabilization of native and artificial protein acceptors *in vivo* and *in vitro* (reviewed in Ciechanover, 1994). Furthermore, these stabilized proteins lacked the multiUb chains present in wild type strains, suggesting an association between multiubiquitination and protein degradation. Moreover, even when Ub-protein conjugation occurs, mutations in specific subunits of the 26S proteasome (Driscoll and Goldberg, 1990; Heinemeyer *et al.*, 1991; ) or inhibition of protease activity (Rock *et al.*, 1994; Vinitsky *et al.*, 1994; Figueiredo-Pereira *et al.*, 1994) resulted in the accumulation and stabilization of Ub-protein conjugates. Furthermore, such strains exhibit stress sensitive phenotypes, cell cycle arrest, or are inviable. Therefore, it is the degradation of these Ub-protein conjugates which is necessary for Ub system functions, not simply ubiquitination.

### 1.3. Enzymes of the Yeast Ub Conjugation System.

**a. The Ubiquitin activating enzyme (E1).**

The *UBA1* gene encodes for *S. cerevisiae* E1 (McGrath *et al.*, 1991). The *UBA1* gene product is a 114 kDa protein. The *S. cerevisiae* E1 protein is highly homologous to E1 in human, mouse, and wheat. The sequence homology apparently extends to functional homology, as the expression of human E1 can complement for a mammalian temperature sensitive E1 cell line (Ayusawa *et al.*, 1992; Handley-Gearhart *et al.*, 1994a).

E1 proteins from different organisms have a putative nuclear localization signal, similar to that found in the nuclear MATα2 protein. Immunolocalization of human E1 in HeLa tissue culture cells demonstrated that the E1 protein is present in the cytoplasm and the nucleus (Handley-Gearhart *et al.*, 1994b). Localization was shown to vary during the cell cycle, however, the significance of the observed migration of human E1 during the cell cycle is not understood.

The *UBA1* gene is essential in *S. cerevisiae* (McGrath *et al.*, 1991) as are its homologs in mouse and human, demonstrating that formation of Ub-protein conjugates is essential for cell viability. Temperature sensitive E1 cell lines [ts85, (Finley *et al.*, 1984) and ts20, (Kulka *et al.*, 1988)] were the original focus of investigations into the role of Ub-protein conjugation. These strains were defective for conjugation, leading to target protein stabilization (Chowdary *et al.*, 1994, Ciechanover *et al.*, 1984). Furthermore, these cell lines arrested at G2/M at nonpermissive temperatures, implicating the Ub system in cell cycle progression (Kulka *et al.*, 1988).

**b. The Ubiquitin-conjugating enzymes (E2s).**

Unlike the single essential E1 enzyme, the E2, or Ub-conjugating (UBC) class consists of at least 10 known E2 proteins (Figure 1.4). As for the E1 enzyme, numerous E2 homologs have been identified in other model systems, including mammals (Plon *et al.*, 1993; Koken *et al.*, 1991a and b; Kaiser *et al.*, 1994a and b; Blumenfeld *et al.*, 1994), *Drosophila melanogaster* (Treier *et al.*, 1992), *Caenorhabditis elegans* (Zhen *et al.*, 1993), *Arabadopsis thaliansis* (Sullivan *et al.*, 1993), wheat (Van Nocker and Vierstra, 1991), and *Candidas albicans* (Damagnez *et al.*, 1995) and have shown that this class of Ub-conjugation enzymes is also highly conserved amongst eukaryotes.

The E2 enzymes are structurally and functionally conserved. There is a core of homology, which includes the active site cysteine apparent upon sequence alignment of different yeast E2s (see Figure 4.1 for sequence alignment of several yeast E2s). Several E2 proteins consist only of this conserved core domain, existing as ~ 14 kDa proteins (Seufert and Jentsch, 1990). Comparison of crystal structures of this domain in two E2s

having unrelated intracellular functions and from evolutionarily distant eukaryotes (Cook *et al.*, 1993; Cook *et al.*, 1992) reveals a stunning conservation in overall 3-dimensional structure (see Chapter 4, Figure 4.2) in an $\alpha/\beta$ motif with four $\alpha$-helices and a four-stranded antiparallel $\beta$-sheet (Cook *et al.*, 1993).

As discussed above, the function of the E2 enzyme is to accept Ub from E1, and then transfer the activated Ub monomer to either an acceptor protein or to an E3. This common function is reflected by the similarity in the core sequences (and apparent structure) of these enzymes. However, two E2 enzymes possess C-terminal sequence extensions necessary for functions specific to that E2 (Silver *et al.*, 1992; Kolman *et al.*, 1992; Raboy and Kulka, 1994; Morrison *et al.*, 1988). It has been suggested that while the homologous core of E2s is necessary for the basic E2 catalytic function and E1 interaction, the additional sequence extensions may be required for target recognition or E3/*trans*-acting factor interactions.

The ability, in yeast, to specifically disrupt a gene encoding an E2 protein has facilitated the analysis of E2 function *in vivo*. The deletion of single E2 genes results in a surprising variety of phenotypes. Different E2 deletions are phenotypically manifested as cell cycle arrest (Goebl *et al.*, 1988), stress sensitivity (Seufert and Jentsch, 1990), defects in DNA repair, induced mutagenesis and sporulation (Jentsch *et al.*, 1987), absence of growth after quiescence (Seufert *et al.*, 1990), or disruption of organelle biogenesis (Wiebel and Kunau, 1992). The phenotypes observed for different E2 disruptions are generally distinct, suggesting different E2s function in discrete cellular pathways. In turn, the lack of functional overlap among E2s may reflect the variation and specificity of proteins targeted for ubiquitination by each E2. In support of this, analysis of purified E2s from reticulocytes using model substrates showed that the E2s differ in their target specificities (Haas *et al.*, 1991).

Despite the obvious differences in E2 function, there is likely some overlap in E2 target recognition. This is supported by the identification of a family of three E2s (UBC1, UBC4 and UBC5) none of which are uniquely essential, while disruption of all three genes is lethal (reviewed in Seufert and Jentsch, 1991).

**c. The Ubiquitin-ligase enzymes (E3s).**

The Ub-ligase enzymes are thought to provide target specificity to the E2 by E3-dependent recognition of the protein substrate. Only two E3 genes have been isolated so far, one encoding the *UBR1* gene in yeast (Bartel *et al.*, 1990) and the second encoding for E6-AP in a human cell line (Huibregtse *et al.*, 1993). The *UBR1* gene produces a 225 kDa protein, homologous to the E3$\alpha$ protein previously isolated from reticulocyte extracts

(Heller and Hershko, 1990), but with no sequence homology to other yeast genes. While these E3s have different protein targets, it has been suggested that they can act as a bridge by binding substrate proteins and specific E2s (reviewed in Varshavsky, 1992).

There is biochemical evidence for other yeast E3s. In *S. cerevisiae* , two other E3-like activities have been biochemically described (Sharon *et al.*, 1991; Parag *et al.*, 1993) but the genes have not been isolated. Enriched lysate fractions, when combined with different E2s, have been shown to allow *in vitro* recognition and ubiquitination of unique cellular proteins, and presumably contain E3s. Database searches of yeast genomic sequences have revealed a yeast sequence homologous to a human E3, RSP5 (Huibragtse *et al.*, 1995). Like E6-AP, there is C-terminal homology including a cysteine residue. To date, the involvement of RSP5 in protein target recognition, or association with different E2s, has not been demonstrated.

Recently, putative E3 activities have been characterized in several other eukaryotes, including rat (Wing and Jain, 1995), clam oocytes (Hershko *et al.*, 1994), rabbit (Blumenfeld *et al.*, 1994), and wheat (Girod *et al.*, 1993a). In many cases, only a biochemical activity has been isolated and therefore it is not known if there is sequence homology between these potential E3s and either yeast N-recognin (UBR1) or human E6-AP. Until the genes encoding these protein factors have been isolated, it remains to be determined if these proteins have structural or functional homology to previously identified E3s.

**d. E2 *trans*-acting factors.**

Other factors known to be involved in E2 target recognition have been identified and the genes cloned, which have no sequence homology to either E6-AP or N-recognin. This class of protein factors have been referred to as *trans*-acting factors. Examples of candidate *trans*-acting factors identified in *S. cerevisiae* include the yeast mating pheromone response pathway protein SST2, (Madura and Varshavsky, 1994), and the DNA-repair protein, RAD18 (Bailly *et al.*, 1994). These proteins have unrelated primary enzymatic functions yet have been implicated in target recognition and E2 interactions. This expanding class of proteins do not have obvious similarities in size, structure, or enzymatic activity. The function of these proteins may be to bridge target proteins and E2s, or alternatively to recruit E2s to sites of activity through protein interactions between the E2- and *trans*-acting factor. At least for the case of RAD18, it has been hypothesized that the physical associations with RAD6 may influence the intracellular location of this E2 to sites of DNA damage through the DNA binding ability of RAD18 (Bailly *et al.*, 1994).

**e. Ubiquitin proteases.**

Regulating the formation of multiUb chains and target protein ubiquitination is the abundant class (>14 to date) of Ub system enzymes known as Ub proteases (Ubps), or Ub-C-terminal hydrolases (UCHs). These are thiol proteases that cleave linear or branched Ub-conjugates, and remove multiUb chains from targeted substrates (reviewed in Hochstrasser, 1995). Recently, several potential Ub protease proteins have been identified in yeast by sequence homology. Ubp proteins carry two common domains, including an active site cysteine residue and two C-terminal histidine residues, separated by 8 or 9 amino acids. Mutations in either of these domains disrupt Ubp activity (Papa and Hochstrasser, 1993). The Ubps differ in their ability to cleave isopeptide bonds between Ub and either small or large protein substrates, perhaps due to differing efficiencies of protein conformation recognition. Mammalian homologues of yeast Ubps have been identified which can cleave either linear or branched Ub conjugates (Wilkinson *et al.*, 1989; Jonnalagadda *et al*, 1987; Hadari *et al.*, 1992). Therefore, like the E1, E2 and E3 classes of Ub system enzymes, the Ub proteases are also apparently conserved amongst eukaryotes.

These enzymes are necessary for three important functions in the Ub system. First, these enzymes are necessary for processing the primary *UBI1*, *UBI2*, *UBI3* and *UBI4* translation products into free Ub proteins and ribosomal subunits (see above). Secondly, they are necessary to maintain free Ub pools *in vivo* via the release of conjugated Ub from either multiUb chains or the release of chains from targeted proteins. A Ub derivative which can become conjugated, but not removed (Hodgins *et al.*, 1992) accumulates Ub-conjugates and depletes free Ub pools, resulting in a cellular phenotype similar to the *UBI4* deletion strain. Secondly, deletion of several Ubp genes results in a similar stress sensitive phenotype which can be alleviated by overexpression of monomeric Ub (R. T. Baker, unpublished results). The third function of Ub proteases is in the regulation of target protein proteolysis, where the removal of the multiUb chains from protein substrates effectively removes the degradative signal recognized by the 26S proteasome and stabilizes the protein target (Papa and Hochstrasser, 1993).

## 1.4. Protein Target Selection.

A key question regarding the Ub dependent proteolytic system is how both native and abnormal proteins are recognized for ubiquitination and degradation. Recently, significant inroads have been made towards understanding how the E2 and E3 proteins may confer selectivity and specificity. As more protein targets are identified, common sequences, structural motifs or postranslational modifications are being correlated with E2

or E3 recognition. Only now as the crystal structures of both E2s and protein targets are being solved can surface residues or clusters which are necessary for target protein recognition by the classes of Ub system enzymes be identified. The following sections will review the current understanding of how the Ub conjugation pathway enzymes allow for selection of protein targets.

In *S. cerevisiae,* at least ten Ub-conjugating (E2) genes have been identified. Individual deletions of these genes has demonstrated that E2s carry out a variety of functions *in vivo*. The RAD6/UBC2 protein is necessary for DNA repair, induced mutagenesis, and sporulation (Prakash, 1989; Jentsch *et al.*, 1992), while the essential CDC34/UBC3 protein is involved in the G1 to S phase transition of the yeast cell cycle (Goebl *et al.*, 1988). The family of E2s, UBC1, UBC4 and UBC5 have overlapping and complementary functions in response to stresses such as heat shock and growth in the presence of amino acid analogs (Seufert and Jentsch, 1990; Seufert *et al.*, 1990). The deletion of the UBC6 and UBC8 genes produced no detectable phenotype (Qin *et al.*, 1991). UBC7 is involved in cadmium resistance (Jungmann *et al*, 1993; Vassal *et al.*, 1992). The UBC9 gene is essential, and is required for the G2/M transition (Seufert *et al.*, 1995). The UBC10 protein is required for peroxisome biogenesis (Wiebel and Kanau, 1992).

**a. E2 Specificity.**

*i. Different functions reflect different targets.* While natural substrates for E2s have rarely been identified, *in vitro* studies using artificial protein targets have conclusively shown that E2s can recognize and target discrete proteins (Haas *et al.*, 1991; Ciechanover, 1994). However, the identification of natural protein targets *in vivo* has revealed that a single protein can be targeted by more than one E2. The MAT$\alpha$2 protein is a rapidly degraded transcriptional regulator, and its stability is affected by four E2 proteins, UBC4, UBC5, UBC6 and UBC7 (Chen *et al.*, 1993). In a similar manner, the GCN4 transcriptional repressor has also been found to be targeted by two E2s, CDC34 and RAD6 (Kornitzer *et al.*, 1994).

Recent evidence also suggests that specific functions of E2s can be attributed to specific domains in the protein. For example, the C-terminus of CDC34 is necessary for its cell cycle function (Silver *et al.*, 1992; Kolman *et al.*, 1992) and may mediate the recognition of the protein targets necessary for G1 to S progression (Silver *et al.*, 1992; Kolman *et al.*, 1992). In support of this, the cell cycle function of CDC34 is transferable to another E2 via its C-terminal domain. Similarly, the C-terminus of RAD6 is essential for

the sporulation function of this E2 such that yeast strains in which this domain is deleted (RAD6Δ) are unable to sporulate (Morrison *et al.*, 1988; Kaiser *et al.*, 1994a).

*ii. E2-E2 interactions.* There is evidence to suggest that E2s can interact either with themselves or with different E2s to form homo or heterodimers. CDC34 and UBC4, both of which are themselves ubiquitinated, have been shown to form homodimers (Silver *et al.*, 1992; Ptak *et al.*, 1994; Gwozd *et al.*, 1995). Additionally, UBC7-UBC7 and UBC6-UBC7 interactions have been demonstrated using the 2-hybrid system (Chen *et al.*, 1993). One hypothesis is that different E2-E2 combinations result in the formation of distinct ubiquitination complexes which have an expanded ability to recognize protein targets. In this way, variations in E2 combinations may well allow for substrate recognition and ubiquitination of a range of substrates. Interestingly, it has been demonstrated that the concentrations of E2s fluctuate in response to cell differentiation (Pickart and Vella, 1988; Genschik *et al.*, 1995), and to heat and stress conditions (Seufert and Jentsch, 1991; Jungmann *et al.*, 1993). Therefore, the ratio and abundance of different E2-E2 associations may be regulated and in turn regulate the subsequent recognition of proteins by these complexes.

*iii. E3 dependence.* While RAD6 and CDC34 are capable of ubiquitination of proteins *in vitro*, (Haas *et al.*, 1991) some E2s clearly require the presence of E3s to ubiquitinate protein substrates and carry out their diverse functions. For example, RAD6 has also been shown to have E3-dependent functions distinct from the sporulation function (Sharon *et al.*, 1991) demonstrating that E2s differ in their E3 dependence which may reflect a change in target protein recognition mediated by E3 associations. Furthermore, the UBC4 protein has been found to require additional, as yet unidentified protein factors for all target protein recognition (Parag *et al.*, 1993) and apparently has no E3-independent functions. These observations have been extended to UBC4 homologs in rat (Wing and Jain, 1995), wheat (Girod *et al.*, 1993a) and *C. elegans* (Zhen *et al.*, 1993).

**b. E3 Selectivity.**

*i. E2-E3 interactions.* E3s may interact with specific domains of E2 proteins to allow for correct protein recognition and subsequent ubiquitination. The yeast E3 protein, N-recognin (UBR1), has been shown to recognize the free N-terminus of proteins to be degraded (reviewed in Varshavsky, 1992). The N-recognin protein interacts by its C-terminus with C-terminal sequences of the 20 kDa RAD6 protein (Madura *et al.*, 1993). The N-recognin association with RAD6 is specific, as N-recognin does not apparently

associate with other yeast E2s (Wing and Jain, 1995; Gonen *et al.*, 1994; Hershko *et al.*, 1994; Parag *et al.*, 1993; Blumenfeld *et al.*, 1994; Sharon *et al.*, 1991).

*ii. Different E2-E3 combinations may allow for different target selection.* Further evidence for specific E2-E3 interactions arose from an investigation of RAD6 point mutants whose surface residues had been systematically mutated (McDonough *et al.*, 1995). The RAD6 mutants were found to be specifically defective in some, but not all, RAD6 functions, suggesting that the mutations introduced had disrupted specific protein-protein interactions. It was hypothesized the mutations prevented individual E2-E3 interactions. In turn, this suggested that individual functions of the E2s required distinct E3 interactions.

## 1.5. Ubiquitination/Degradation Signals.

A major question in biological systems is the manner in which proteins are selected for degradation. Targeting of proteins for degradation requires both diverse signals and distinct enzymatic factors. The ubiquitin system affects the half lives of extremely diverse classes of intracellular proteins, including both short-lived regulatory proteins, abnormal or unfolded proteins, as well as membrane bound proteins (reviewed in Ciechanover and Schwartz, 1994). Furthermore, the Ub degradative system affects proteins in cytosolic, nuclear and vacuolar (lysosomal) compartments (Ciechanover *et al.*, 1991b; Gropper *et al.*, 1991). In some cases, degradation is cell cycle dependent, ligand dependent, and in rare cases, examples of partial proteolysis have been documented. However, no single molecular determinant has been discovered which is sufficient to signal a protein for ubiquitination and subsequent degradation (reviewed in Ciechanover and Schwartz, 1994). To be discussed are several recognition determinants for Ub dependent proteolysis of cellular proteins such as single amino acid determinants, sequence motifs, and postranslational modifications.

### a. The role of the amino-terminal residue.

Perhaps the best characterized molecular determinant for degradation by the Ub dependent system is the free amino terminal residue. There are several reviews covering this topic (Varshavsky, 1992; Arfin and Bradshaw, 1988; Finley, 1992; Jentsch, 1992). Proteins are targeted for conjugation to Ub by the free N-terminal amino acid, following what is referred to as the N-end rule (Gonda *et al.*, 1989; Bachmair *et al.*, 1986). The specificity of the amino-terminal targeting system is defined by the yeast E3, N-recognin (UBR1) in combination with RAD6. Experiments by Bachmair *et al* (1986) had elegantly

demonstrated that free amino terminal residues on target proteins with both basic and bulky hydrophobic groups are significantly destabilized by this system. The N-end rule has also been shown to hold true in higher eukaryotes (Kaiser *et al.*, 1994a; Reiss *et al.*, 1988; Gonda *et al.*, 1989), where, in addition to the N-recognin homolog E3α, a second E3, referred to E3β, destabilizes proteins with residues other than those previously mentioned (Heller and Hershko, 1990).

While several artificial (Bachmair *et al.*, 1986; Gonda *et al.*, 1989) and natural (Madura and Varshavsky, 1994; deGroot *et al.*, 1992) targets have been identified as using the N-end rule, it is clear that N-end dependent targeting is not a major pathway for target recognition, and its function may involve only limited subsets of protein substrates. In support of this, deletion of the yeast *UBR1* gene is not lethal (Varshavsky, 1992; Bartel *et al.*, 1990), and numerous proteins are still degraded *in vivo* in strains lacking the gene. Secondly, approximately 80% of cellular proteins do not have free amino termini, and instead carry $N^{\alpha}$acetyl groups which effectively block the N-terminus (Brown and Roberts, 1976). The following sections discuss less well characterized signals for degradation which appear to result in specific recognition of the targets for ubiquitination and subsequent degradation.

**b. $N^{\alpha}$-acetylation**

The vast majority of cellular proteins do not have free amino termini and are not efficient substrates for the N-end rule pathway. Nevertheless, some $N^{\alpha}$-acetylated proteins are targeted for ubiquitination by as yet unknown Ub ligases (E3s). These ubiquitinated proteins are degraded by the 26S protease (Gonen *et al.*, 1992), but degradation is dependent on the presence of the recently identified protein synthesis elongation factor, EF-1α (Gonen *et al.*, 1994). However, unlike the N-recognin component associated with RAD6 for recognition of free amino terminal residues, the EF-1α factor is not involved in the formation of Ub-protein conjugates, but instead functions with the 26S protease to stimulate degradation. It was hypothesized that this factor recognized and associated with the Ub-protein conjugate as a necessary step before degradation (Gonen *et al.*, 1994).

While the combined action of purified E1, E2 (rabbit), E3α and EF-1α is necessary for the *in vitro* degradation of numerous model $N^{\alpha}$acetylated protein substrates (Gonen *et al.*, 1994), it is still undetermined if the majority of proteins are recognized, ubiquitinated, and degraded in a similar manner. Further investigations should provide insights as to how the $N^{\alpha}$acetyl group acts as a degradation signal, and how other acetylated proteins escape degradation.

**c. Abnormal or unfolded proteins.**

A major class of proteins targeted for ubiquitination are abnormal or unfolded proteins due to thermal denaturation, amino acid analog incorporation, or mistranslation (Ananthan *et al.*, 1986). Under conditions of heat stress, DNA damage, or growth in the presence of amino acid analogs, an accumulation of abnormal proteins is observed which are rapidly ubiquitinated and degraded (Burdon *et al.*, 1987; Parag *et al.*, 1987). While not proven, it is thought that the misfolding of these proteins results in the exposure of internal hydrophobic residues which are then recognized by components of the Ub system.

Other cellular proteins specifically recognize misfolded proteins, supporting the idea that there are unique determinants exposed upon misfolding. The chaperonins, or heat shock proteins have been found to specifically recognize, bind, and in some cases repair misfolded proteins in both prokaryotes (Schröder *et al.*, 1993) and eukaryotes (Mifflin and Cohen, 1994). Furthermore, the accumulation of abnormal proteins results in the induction of the stress response, increasing the expression of heat shock proteins (Grant *et al.*, 1989; Ananthan *et al.*, 1986). Interestingly, expression of the polyUb gene in yeast, (*UBI4*, Finley *et al.*, 1987) and other eukaryotes (Lee *et al.*, 1988; Swindle *et al.*, 1988) as well as two yeast E2 genes, *UBC4* and *UBC5*, (Seufert and Jentsch, 1990) is induced under stress conditions. In addition, deletion of *UBC4/5* or *UBI4* both result in stress sensitive phenotypes (Seufert and Jentsch, 1990; Finley *et al.*, 1987).

**d. Destruction boxes.**

One motif present in several proteins subject to Ub dependent degradation is the destruction box. Destruction boxes encompass both a short (nine amino acids) conserved amino acid sequence followed downstream by a stretch of sequence rich in lysines which are thought to be targeted for ubiquitination. Destruction boxes were first identified in sea urchin mitotic (B) cyclins (reviewed in Hunt, 1991), and the motif (RXALGXIXN) was shown to be required for their proteolysis (Glotzer *et al.*, 1991; Tyers *et al.*, 1992). The destruction box motif has also been identified in an unrelated yeast protein, the yeast uracil permease. In agreement with the observation of mutations in cyclin destruction boxes, a similar mutation in the uracil permease protein also results in stabilization under conditions previously leading to degradation (Galan *et al*, 1994).

**e. Protein conformation-dependent Ubiquitination.**

This section presents current evidence gathered that suggests recognition of some cellular proteins is conformation dependent. Previous work has suggested that cell surface receptors undergo conformational changes upon ligand binding. Recently, however, it has

been determined that several transmembrane receptors become rapidly, and efficiently ubiquitinated only upon ligand binding on the extracellular surface. Examples include the T cell receptor (Cenciarelli *et al.*, 1992), and the PDGF receptor (Mori *et al.*, 1993). Furthermore, calmodulin is been shown to be ubiquitinated after $Ca^{2+}$ binding (Jennissen *et al.*, 1992; Parag *et al.*, 1993).

So far, only the cause and effect of ubiquitination of these substrates have been observed. It is unknown what molecular determinants are presented in the ligand-bound conformation that allow for recognition by the Ub-conjugation pathway enzymes. At least for the case of calcium-bound calmodulin, it has been demonstrated that a novel E3-like activity is necessary for ubiquitination. It may be, therefore, these diverse proteins associate with E3s whose protein-interactions occur only in the ligand-bound state.

**f. Multiubiquitination of target proteins.**

Aside from signals within protein targets that allow for recognition by Ub system enzymes, the subsequent degradation of ubiquitinated proteins relies on the presence of covalently bound multiUb chains.

*i. K48 multiUb chains.* In 1985, Hershko observed that the number of Ub moieties present on a protein substrate exceeded the number of lysine residues available (Hershko and Heller, 1985). This was perhaps the first indication that Ub was being conjugated to Ub to form chains. Since this time, numerous ubiquitinated proteins have been shown to carry multiUb chains at one or more lysine residues, and that the multiUb chains contain variable numbers of Ub moieties. MultiUb chains were isolated and mapped (Chau *et al.*, 1989; Chen *et al.*, 1991; Van Nocker and Vierstra, 1993), and it was found that these chains consisted of homogenous linkages between the C-terminus of one Ub monomer and the lysine (K) at position 48 of the next. It is now known that these K48 multiUb chains can be made by several E2s in different eukaryotes (Haas *et al.*, 1991; Chen *et al.*, 1991; Van Nocker and Vierstra, 1993). Furthermore, monoclonal antibodies raised against multiUb chains (which do not recognize monomeric Ub) revealed that K48 multiUb chains are prevalent *in vivo* (Fujimuro *et al.*, 1994).

*ii. K48 Ub-Ub chains as a degradative signal.* One of the most significant insights into Ub-dependent degradation was the observation that K48 multiUb chains are necessary for target protein degradation, and that the 26S proteasome contains a subunit that specifically recognizes these Ub-Ub linkages. First, mutation of the lysine 48 residue to a conserved arginine (R) residue prevented the formation of Ub-Ub conjugates *in vitro* (Chau *et al.*, 1989). Significantly, proteins conjugated to R48 Ub were stabilized compared to

their K48 multiubiquitinated relatives. These experiments clearly demonstrated that K48 multiUb chains were involved in target protein degradation.

Secondly, mutations in several yeast 26S proteasome subunits (Hilt *et al.*, 1993a and b; Richter-Ruoff *et al.*, 1992; Fujiwara *et al.*, 1990; Georgatsou *et al.*, 1992; Ghislain *et al.*, 1993) resulted in the stabilization of K48 multiubiquitinated substrates. Furthermore, these proteasome mutants exhibited phenotypes consistent with deletions of other Ub system components, such as cell cycle arrest (Ghislain *et al.*, 1993), stress sensitivity (Heinemeyer *et al.*, 1991; Hilt *et al.*, 1993b) and accumulation of abnormal proteins (Heinemeyer *et al.*, 1991). Lastly, it was also observed that free K48 multiUb chains (when composed of 4 or more Ub monomers) were capable of competing with Ub-protein conjugates for protease associations (Deveraux *et al.*, 1994). Together, these experiments imply that the conjugation of K48 multiUb chains to target proteins, followed by 26S proteasome association are necessary for degradation.

*iii. Alternative Ub-Ub linkages.* Not expected was the observation that in some systems, R48 Ub derivatives were able to form Ub-Ub conjugates at positions other than K48. This was observed both *in vivo* (Johnson *et al.*, 1992) and *in vitro* (Hadari *et al.*, 1992; Gregori *et al.*, 1990). Two different investigations have revealed that the alternative Ub-Ub linkages may have distinct functions *in vivo* . First, multiUb chains created with the R48 Ub derivative were resistance to Ub-protease activity (Hadari *et al.*, 1992; Chen and Pickart, 1990), suggesting that they were structurally distinct from the K48 multiUb chain which is affected by the Ub protease tested. Secondly, the stress sensitive phenotype of the polyUb gene deletion strain (*ubi4Δ*) was restored by the expression of R48 Ub (Finley *et al.*, 1994) implying that the K48 Ub-Ub linkage was not necessary for the stress resistance function of the Ub proteolytic system in *S. cerevisiae*. It may be, therefore, that alternative Ub-Ub conjugates have *in vivo* functions distinct from the K48 degradative signal.

### 1.6. Intracellular roles of Ubiquitination.

#### a. Degradation of Ub-protein conjugates.

The most well understood intracellular role of ubiquitination is to mark proteins for degradation. Normal cell growth requires that key regulatory proteins be degraded at specific points in the cell cycle, several of which have been shown to be turned over in a Ub dependent manner. Also, cell survival under adverse conditions requires a rapid response to the accumulation of abnormal protein accumulation, which has also been shown to require the action of Ub conjugation and proteasome degradation. The

importance of the Ub system for protein degradation was revealed by studies of the mammalian mutant cell line ts85 which harbors a temperature sensitive E1 enzyme (Ciechanover *et al.*, 1984; Finley *et al*,, 1984). At nonpermissive temperatures, ts85 cells exhibit strong defects in the degradation of otherwise short lived or abnormal (amino acid analog containing) protein, suggesting that a significant portion of bulk protein degradation is mediated by the Ub system (Ciechanover *et al.*, 1984).

Significantly, a new aspect of Ub dependent degradation has come to light from recent studies, namely that of partial degradation. Studies of p105 degradation to yield the transcription factor NFκB have shown that, despite the ubiquitination of p105, proteolytic degradation resulted in partial proteolysis, similar to the cleavage of the signal peptide from protein precursors upon reaching the mitochondria, to yield the NFκB protein (Palombella *et al.*, 1994). Secondly, there is accumulated evidence to suggest that the Ub dependent pathway is intimately involved in antigen processing for MHC class I presentation (reviewed in Monaco, 1995). In this case, Ub dependent 26S proteolysis does not result in free amino acids, but polypeptides of 8-9 amino acids in length (Rammensee *et al.*, 1993).

**b. DNA repair.**

The RAD6 Ub-conjugating enzyme, in conjunction with other DNA repair enzymes, is responsible for a substantial fraction of yeast resistance to DNA damage after exposure to UV light. A RAD6 deletion strain exhibits a surprisingly diverse phenotype, including defects in cellular proliferation, sporulation and UV induced DNA damage repair. The role of the Ub system in DNA repair is highlighted by the observation that both the *RAD6* and the *UBI4* (polyUb) genes are activated in response to DNA damaging agents (Madura *et al.*, 1990; Treger *et al.*, 1988). As for its amino terminal targeting function in conjunction with the E3, N-recognin, the UV induced DNA repair function of RAD6 is carried out in association with an additional protein factor, RAD18. Both the RAD6 and RAD18 proteins are part of the same epistasis group of error prone DNA repair. Recently, it was shown that RAD6 and RAD18 proteins physically interact and do so through RAD6 domains distinct from that necessary for UBR1 interactions (Bailly *et al.*, 1994).

The RAD6 dependent DNA repair activities are likley regulated by the ubiquitination of target proteins. A RAD6 mutant with an inactivated active site cysteine (Sung *et al.*, 1990) was unable to complement for a *RAD6* deletion strain, clearly demonstrating that RAD6 function in DNA repair, sporulation and cell proliferation are dependent on its ability to form Ub-protein conjugates. Identification of proteins targeted by RAD6 and the consequences of their modification on DNA repair are not yet known.

**c. Transcription.**

Transcriptional regulation of genes is a way of controlling the intracellular protein levels, but it has now been demonstrated that the levels of several regulators are themselves controlled by Ub-dependent proteolysis (Palombella *et al.*, 1994; Kornitzer *et al.*, 1994; Chowdary *et al.*, 1994; Chen *et al.*, 1993; Hochstrasser and Varshavsky, 1990; Treier *et al.*, 1994). The first regulatory factor to be identified was the short lived yeast MATα2 transcriptional repressor (Hochstrasser and Varshavsky, 1990), and its degradation was shown to be dependent on at least four E2s, including UBC4, UBC5, UBC6 and UBC7 (Chen *et al.*, 1993). Next, the yeast GCN4 transcriptional activator, involved in amino acid and purine biosynthesis, is a short lived protein which is targeted by the Ub system for degradation via a domain rich in proline-glutamic acid-serine-threonine (PEST) sequences (Kornitzer *et al.*, 1994). These PEST domains had previously been found in other short-lived cytosolic proteins and have been hypothesized to function as degradation signals (Rogers *et al.*, 1986).

Most unusual, perhaps, is the partial proteolytic processing of the NFκB precursor, a step required for its activation (Palombella *et al.*, 1994). This transcription factor is necessary for the expression of numerous genes involved in immune and inflammatory responses (reviewed in Grilli *et al*, 1993). Perturbation of proteasome subunits inhibit precursor processing *in vivo*, but the identity of the Ub-conjugating enzymes necessary for recognition have not been identified (Palombella *et al.*, 1994).

**d. The yeast stress response.**

Ub-dependent protein degradation is an essential function of the eukaryotic stress response (Burdon *et al.*, 1987). Stress conditions arising from heat (thermal denaturation; Parag *et al.*, 1987), amino acid analogs (abnormal peptide incorporation; Menninger *et al.*, 1994), or errors in translation (Grant *et al.*, 1989) generally result in the accumulation of misfolded and abnormal proteins. The function of the Ub system in the stress response is thought to be to clear the cell of such proteins before toxic levels are reached to allow for cell viability under these adverse conditions (Haas and Bright, 1985; Shang and Taylor, 1995).

Several genes involved in bulk protein degradation and stress resistance have been identified and characterized in yeast. PolyUb genes from various eukaryotes (Fraser *et al.*, 1991; Genschik *et al.*, 1994; ), including the ***UBI4*** gene in yeast (Finley *et al.*, 1987; Cheng *et al.*, 1994; Treger *et al.*, 1988), are induced in response to the various stresses mentioned above. Deletion of the ***UBI4*** gene results in extreme stress sensitivity, implicating the necessity for ubiquitination in resistance to stress (Finley *et al.*, 1987).

Three additional genes, coding for the E2 enzymes UBC1, UBC4 and UBC5 are individually dispensable, but together constitute an essential protein group, indicating that their functions overlap (Seufert *et al.*, 1990). *UBC1* is predominantly expressed in stationary cells and can only partially complement for the combined deletion of the *UBC4* and *UBC5* (*UBC4/5*) genes (Seufert *et al.*, 1990). UBC4/5 encode almost identical enzymes (Seufert and Jentsch, 1990).

Significantly, the deletion of the stress resistance genes *UBC4* and *UBC5* results in a yeast strain that is severely retarded in cell proliferation, as well as exhibiting extreme sensitivity to heat stress or growth in the presence of amino acid analogs (Seufert and Jentsch, 1990) similar to the sensitivities observed for the *UBI4* deletion strain. *UBC4/5* deletion mutants are noticeably deficient in the degradation of otherwise long-lived and abnormal proteins, a fact revealed by pulse-chase analysis (Seufert and Jentsch, 1990). Additionally, the *UBC4/5* deletion strain constitutively expresses the major heat shock proteins, suggesting that defects in degradation of abnormal protein results in the accumulation of abnormal proteins, which is known to induce the stress response (Ananthan *et al.*, 1986).

The UBC4 and UBC5 E2s are necessary for stress resistance in yeast, and their function is presumed to be to target and ubiquitinate the majority of abnormal proteins generated during stress conditions. A key question remaining is the means whereby the UBC4 and UBC5 proteins recognize this general class of abnormal and misfolded proteins. UBC4/5 are E3-dependent, (or *trans*-acting factor dependent) for target protein recognition (Wing and Jain, 1995; Girod *et al.*, 1993a), and until these associated proteins can be identified for UBC4 function, this question will remain. The UBC4/5 E2s are highly conserved in other eukaryotes, as evidenced by the number of organisms which have been found to encode homologs, including rat (Wing and Jain, 1995), *C. albicans* and *S. pombe* (Damagnez *et al.*, 1995), wheat (Girod *et al.*, 1993a and b), *Drosophila* (Treier *et al.*, 1992), *C. elegans* (Zhen *et al.*, 1993), and *Arabadopsis* (Girod *et al.*, 1993b). It is likely that the UBC4/5 proteins carry out similar stress resistant functions in these organisms as the UBC4/5 deletion strain phenotypes can be fully complemented by the expression of a UBC4 homolog *in vivo* (Treier *et al.*, 1992; Zhen *et al.*, 1993).

In summary, the combination of genetic and biochemical analysis of *S. cerevisiae* has advanced our understanding of the Ub degradation system. In turn, this understanding has demonstrated that this regulated degradation pathway is more complex than first imagined. While the mechanism of Ub conjugation has been biochemically described, the regulation of target selection, ubiquitination, and degradation are only beginning to be

understood. The multitude of diverse determinants recognized in different protein targets by either E2s, or in combination with E3s or *trans*-acting factors, infers that the Ub system for protein degradation is highly flexible. Lastly, the conservation of each class of protein associated with either Ub conjugation, target recognition, or subsequent degradation in evolutionarily diverse eukaryotes reveals that studies of the Ub system in simple eukaryotes are likely to be directly applicable to higher eukaryotic systems.

## Objectives of the Thesis

The *S. cerevisiae* Ub system has provided a useful tool for the identification of components of the Ub system and characterization of their roles *in vivo*. However, many investigations of the Ub system in yeast have focused on identifying protein targets and determinants of protein target recognition. The material presented in the chapters of this thesis represent the result of individual studies undertaken (i) to identify the sites of alternative Ub-Ub linkages distinct from the canonical K48 multiUb chain, and to identify the Ub-conjugating enzymes necessary for their formation, and also (ii) to investigate the intracellular role(s) of the alternative linkages to determine if they function as a signal distinct from that of the K48 multiUb degradation signal. These investigations were extended by focusing on UBC4, which was capable of creating the alternative linkages. Mutational analysis of UBC4 was done to (iii) determine the dependence of UBC4 function in cell proliferation, and resistance to heat and amino acid analog stress, on highly conserved surface residues. Lastly, to elucidate both target selection and linkage specificity necessary for the various functions of UBC4 *in vivo*, (iv) a genetic screen was used to identify proteins that associate with a UBC4 derivative *in vivo* .

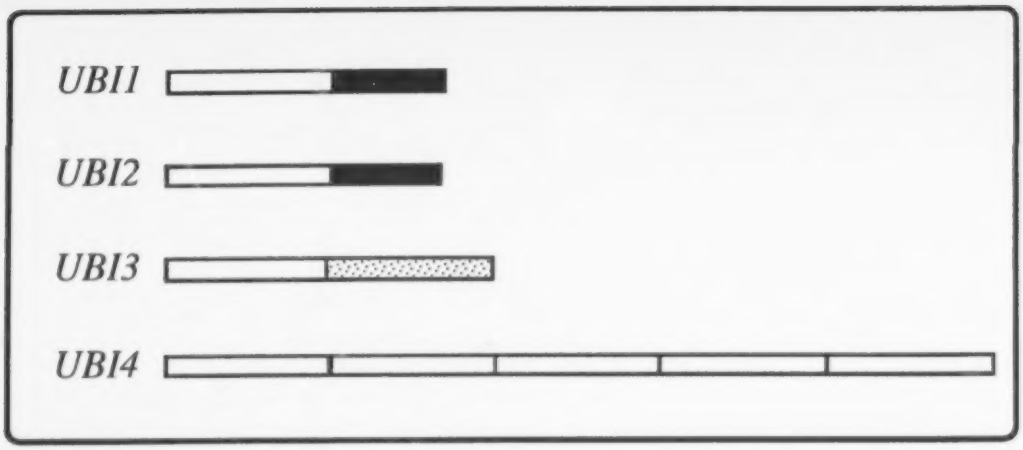


FIGURE 1.1

**The four Ub genes of *S. cerevisiae*.**

The four genes encoding for Ub are shown as white rectangular boxes, with C-terminal fusions appended to their 3' ends. The *UBI1* and *UBI2* genes are fused to identical sequences (dark stippled boxes) which encode for a protein component of the large ribosomal subunit. The *UBI3* gene is fused to sequences encoding a protein subunit, S34, of the small ribosomal subunit (Finley *et al.*, 1989). The *UBI4* gene is also a fusion protein, but encodes for a pentameric, head-to-tail repeat of Ub monomers with no intervening sequences (Finley *et al.*, 1987), as indicated by the five juxtaposed rectangles.

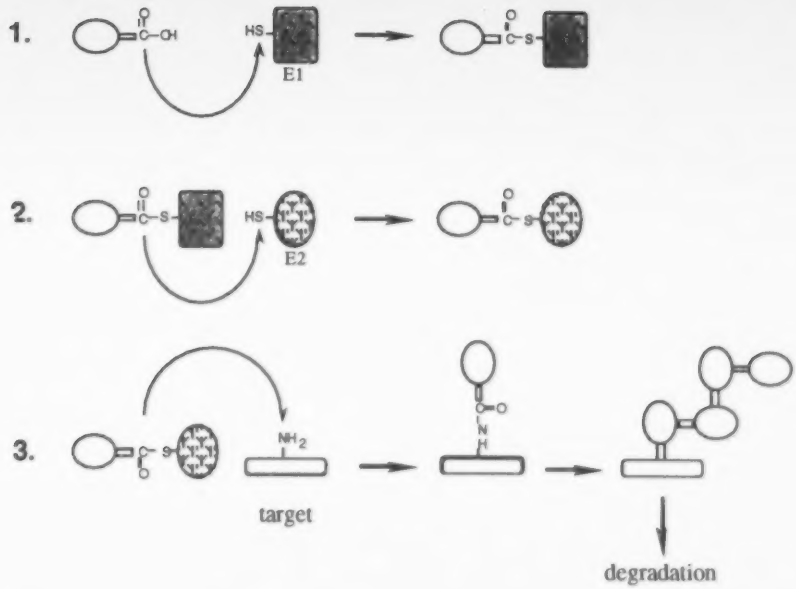


FIGURE 1.2

**The Mechanism of Ub conjugation.**

Shown is a schematic representing the three steps necessary for Ub conjugation. Symbols and nomenclature are as follows. Ub; the Ub protein is represented by a ball-and-stalk structure, with the C-terminal carboxyl group indicated. E1; hatched rectangle with the active site cysteine (-SH) group indicated. E2; patterned oval, also with the active site cysteine (-SH) indicated. Target protein; open rectangle, with an acceptor lysine ε-amino group ($-NH_2$) indicated. **1.** The C-terminus of Ub forms a thioester bond with the active site cysteine of E1 at the expense of ATP. **2.** Ub is transferred to the active site cysteine of an E2 in a transthiolation reaction, forming a Ub-E2 conjugate. **3.** The C-terminus of Ub becomes covalently joined to the ε-amino of the target protein, forming an isopeptide bond. In many instances, the target protein becomes multiubiquitinated, and degraded by the 26S proteasome.

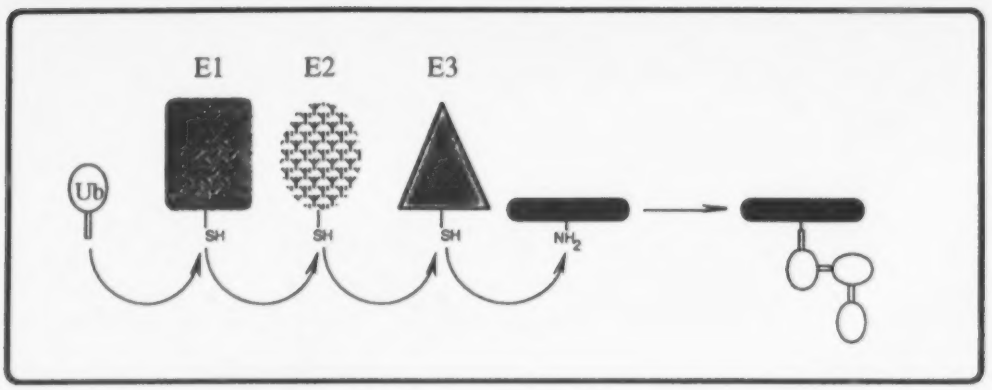


FIGURE 1.3

**E3-dependent Ub conjugation.**

Shown is a schematic representation of a known pathway of E3-dependent conjugation of Ub to target proteins. The symbols and nomenclature are as for Figure 1.2, with one addition. The triangle represents an E3 protein, with the active site cysteine (-SH) group indicated. Ub can be transferred through high energy thiol bonds sequentially from E1, to E2, to E3, to the ε-amino group of the target protein. The E3 protein can form a bridge, by dual recognition, between the E2 and the targeted substrate. Lastly, the subsequent formation of a multiUb chain can result in selective protein degradation of the target protein.

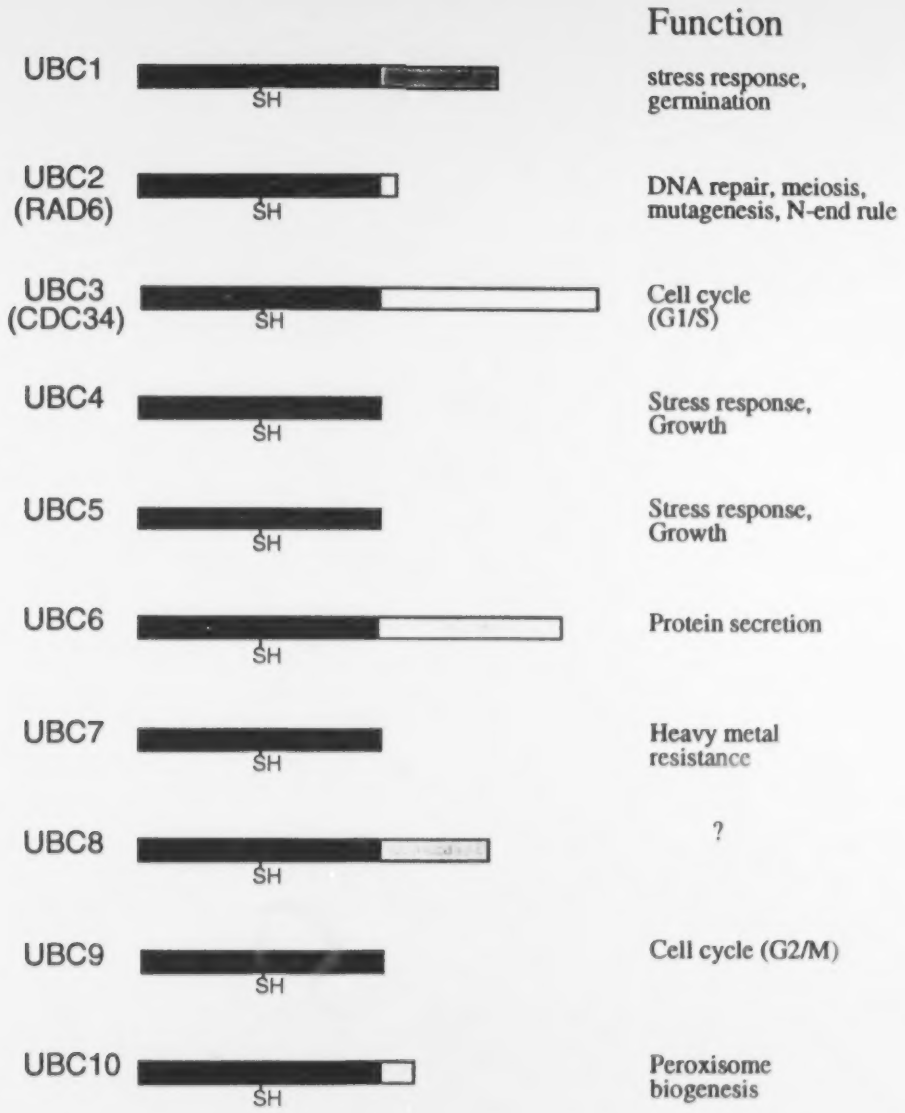


FIGURE 1.4

**The Ub-conjugating enzymes of *S. cerevisiae*.**

A schematic representation of yeast E2s (adapted from Jentsch *et al.*, 1990). Solid boxes for each E2 represent the conserved domain within all E2s, including the active site cysteine (-SH). Open boxes represent acidic C-terminal extensions, and stippled boxes are non-acidic extensions.

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# CHAPTER 2. *In vivo* Determination of Alternative Sites of Ubiquitin-Ubiquitin Conjugation.

## 2.1 Introduction.

The *in vivo* functions of Ub are carried out by its postranslational attachment to selected protein targets (reviewed in Ciechanover, 1994 a and b). Ub forms an isopeptide linkage between its C-terminus and the ε-amino group of lysine residues present in the protein substrate (Chau *et al.*, 1989; Hershko *et al.*, 1988). Proteins have been found attached to a single Ub molecule (Haas *et al.*, 1990), yet the majority of ubiquitinated proteins carry numerous Ub monomers which are sequentially attached to one another through lysine 48 to form K48 multiUb chains (reviewed in Varshavsky, 1992). These multiubiquitinated proteins, moreover, were found to be necessary for degradation (Bachmair *et al.*, 1989; Gonda *et al.*, 1989; Hershko *et al.*, 1991; Gregori *et al.*, 1990). These observations lead to the hypothesis that K48 multiUb chains were serving as a degradation signal for the protein targets (Ellison and Hochstrasser, 1991; Johnson *et al.*, 1992; Hodgins *et al.*, 1992).

Both *in vivo* (Johnson *et al.*, 1992)) and *in vitro* (Gregori *et al.*, 1990; Haas *et al.*, 1991) experiments have clearly demonstrated the appearance of alternative Ub-Ub conjugates created through sites other than lysine 48. However, for these alternative Ub-Ub linkages, neither the identification of the alternative conjugation site was made, nor the function of the alternative linkages determined. Nevertheless, the presence of alternative Ub-Ub linkages suggested that they may act *in vivo* in a manner distinct from that of the K48 proteolytic signal.

In this chapter, the development of an *in vivo* system for the detection and identification of alternative Ub-Ub linkages is described. Specific Ub derivatives were created and used as tools in order to determine the precise sites that are acceptable for alternative Ub-Ub linkages to form. This *in vivo* analysis revealed the presence of three different lysine residues suitable for Ub-Ub conjugate formation, one of which was the canonical K48 Ub-Ub linkage.

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## 2.2 Materials and Methods.

### Plasmids and Yeast Strains.

Each of the Ub derivatives described in Chapter 2 was expressed from a high-copy-number yeast plasmid that is identical to the *TRP1* copper inducible Ub expression plasmid YEp96 (described in Ellison and Hochstrasser, 1991) with the exception of the specific changes noted below. (i) All K-to-R substitutions listed for the various Ub derivatives shown in Figure 2.1-2.5 were made by replacing K codons with the R codon AGA. (ii) The DNA sequence of the N-terminally Myc-tagged Ub (mUb) is identical to that described for YEp105 (Ellison and Hochstrasser, 1991). (iii) The DNA sequence of the mUb.$K_0$ gene is identical to mUb except for the seven K-to-R codons changes shown in Appendix B (Figure B.3, B.5 and B.6). (iv) In genes encoding C-terminally Myc-tagged Ub (Ubm), the Gly76 codon of Ub has been replaced with a DNA segment encoding the peptide sequence APCEQKLISEEDL (containing the Myc epitope). The AP portion of this peptide (occupying amino acid positions 76 and 77) was included to prevent cleavage by the Ub processing enzymes (Johnson *et al.*, 1992). (v) In UbΔ genes, the C-terminal codons Gly75 and Gly76 have been deleted. (vi) The donor Ub derivatives used for Figures 2.1 and 2.2 are contained on a common high-copy-number *URA3* vector that was created by positioning the *Bam*HI-*Pst*I expression cassette from the YEp96 (Ub), YEp105 (mUb) (Ellison and Hochstrasser, 1991), or the mUb.$K_0$ equivalent of the YEp105 between the *BamH*I and *Pst*I sites of the high-copy *URA3* plasmid YEp352 (Hill *et al.*, 1986). The accompanying *TRP1* sequence of the cassette was subsequently removed from the resulting vector as a *Cla*I-*Nar*I fragment and the plasmid was religated. The DNA sequences of all plasmid borne Ub genes were verified by DNA sequencing with an automated DNA sequencer (Applied Biosystems operated by the Department of Biochemistry DNA Sequencing and Synthesis Facility at the University of Alberta).

Schematic representations of these expression plasmid are shown in Figure 2.1 and the strategy used in their construction in outlined in Appendix A. Plasmids were transformed either singly or in the combinations specified in Figures 2.1-2.5 into the yeast strain SUB60 (*MAT***a***; lys2-801; leu2-3,2-112; ura3-52; his3-Δ200; trp1-1(am); ubi4-Δ2::LEU2* ). SUB60 was a gift of D. Finley (Finley *et al.*, 1987). SUB60 carries a deletion for the polyUb gene, *UBI4*.

### Expression of Ub derivatives in Yeast.

25 ml cultures of SUB60 inoculated from freshly transformed yeast colonies were grown to mid-log phase overnight at 30°C in synthetic defined (SD) media supplemented

with selected amino acids critical for growth (including 10 mg/L histidine, 40 mg/L lysine and either tryptophan or uracil depending on plasmid selection). The next morning, cultures were diluted to 3.0 x $10^6$ cells per ml in a 25 ml volume SD. To this, 250 µl of 100x $CuSO_4$ (100 µM final concentration) was added to induce the expression of plasmid Ub from the copper inducible *CUP1* promoter. Cells were grown for a minimum of 2 generations to a maximum of 5 generations before harvesting. Yeast media growth conditions and transformation protocols are detailed in Appendix A.

**Purification of Ub and Ub-Ub conjugates.**

SUB60 coexpressing mUb in combination with either UbΔ or UbR48Δ were grown and induced for Ub synthesis as described above in the presence of 3 µCi/ml [$^{14}$C]lysine throughout the induction period (2 generations).

a. Spheroplast formation: Following induction, cells were pelleted and resuspended in 500 µl of 1M sorbitol, 1 mM DTT, and 5 mg/ml zymolyase (Kirin Brewery, Japan). Cells were incubated at 37°C until a high proportion of cells rapidly lysed in milliQ water as observed under a light microscope. At this time, cells were gently pelleted (500 x g, 1 min) and the supernatant was removed. To lyse the cells, 450 µl of milliQ water and 50 µl of 10 x protease inhibitor cocktail (see Appendix C) was vortexed into the cell pellet. Cellular debris was pelleted (17,000 x g, 10 min, 4°C) and the supernatant was transferred to a fresh tube.

b. Selective Acid precipitation of Ub and Ub conjugates from yeast lysates: To 500 µl of yeast lysates from $CuSO_4$ induced cells, 50 µl of 1 N HCl was rapidly added and the tubes placed on ice for 20 minutes. The precipitate was pelleted by centrifugation (17,000 x g, 10 min, 4°C). The supernatant (containing Ub) was precipitated by the addition of 25% (v/v) of 100% trichloroacetic acid (TCA). To precipitate, 1 µl of a 10 mg/ml BSA carrier protein was mixed in prior to TCA addition, and the final mixture was placed on ice for 30 minutes before centrifugation (17,000 x g, 30 min, 4°C). The resulting pellets were washed in acetone to raise the pH before loading on a polyacrylamide gel. A convenient volume of 2x SDS sample buffer was added, and if too acidic, Tris base crystals were added until the sample buffer turned blue.

Samples were separated on an 18% acrylamide gel and the proteins were then transferred to Immobilon-P membrane by electroblotting using CAPS buffer (10 mM CAPS ((3-[cyclohexoamino]-1-propanesulfonic acid) [Sigma]), 10% methanol, pH 11). To detect the monomeric forms of Ub and mycUb as well as the Ub conjugates, the membrane was briefly stained with Ponceau S (0.5%) in acetic acid (1%). The protein band(s) of interest (mUb-UbΔ, mUb-UbR48Δ, and mUb and UbΔ monomers) were

excised from the membrane with a razor, cut into 2 $mm^2$ fragments and put into a 0.5 ml eppendorf tube.

**Cyanogen Bromide (CNBr) Cleavage.**

To cleave the protein, 150 µl of 0.15M CNBr in 70% formic acid was added and allowed to react overnight at 22°C with gentle agitation in the dark (Matsudaira, 1989). 12 hours later, the excess reagents were removed via vacuum centrifugation in a Speedvac (Savant Instruments). When dry, 50 µl of milliQ water was added, vortexed briefly and redried in the Speedvac, followed by two water addition/removal steps. To remove the proteins from the Immobilon-P membrane for analysis by SDS PAGE, 50 µl of elution buffer (2% SDS, 1% Triton X-100 in 50 mM Tris pH 9.2) was added to the dried Immobilon-P fragments and incubated for 90 minutes at room temperature with gentle agitation. Efficiency of elution was monitored by scintillation counts of the $^{[14C]}$lysine labeled proteins present on the membrane after protein elution (82% and 73% elution respectively for mUb and UbΔ). 6.25% glycerol with 0.01% bromophenol blue was added directly to the eluate and loaded directly onto an 18% acrylamide gel. Following electrophoresis, protein bands were visualized either by Western analysis using an anti-Ub antibody as the probe or by autoradiography. Methods and conditions used for SDS-PAGE and protein detection are elaborated in Appendix A.

## 2.3. Results

### 2.3.1. Ubiquitin-ubiquitin linkages are targeted to at least two sites.

A genetic approach, rather than a purely biochemical approach, was used to identify the site(s) on Ub, other than K48, that are capable of forming an alternative Ub-Ub linkage. We believed that through the use of engineered Ub mutants we could unequivocally determine the site(s) on Ub used to form the alternative Ub-Ub linkages *in vivo* using the yeast *S. cerevisiae*. These mutants were engineered as tools to create a method of identifying sites on Ub used as acceptors for ubiquitination. It was assumed that a logical place to initiate the investigation would be to determine if one or more lysines in Ub was acting as an acceptor for ubiquitination in addition to the lysine at position 48. To determine which lysine(s) was capable of accepting another Ub molecule, it was necessary to create a series of Ub mutants that were specifically mutated in various combinations at the seven lysine residues in Ub. The lysines were converted to arginine because it had been previously shown that the conversion of lysine 48 to arginine prevented the assembly of K48 multiUb chains (Chau *et al.*, 1989), yet this conservative replacement would minimize the perturbation of protein structure as compared to a nonconservative

replacement. By reducing the number of available lysines in Ub, identification of those residues which can function as acceptors for ubiquitination should be considerably simplified.

To identify the lysines used in isopeptide bond formation between two Ub proteins, it was considered prudent to further modify the lysine-to-arginine Ub mutants into Ub derivatives that would act as acceptors of Ub exclusively. To accomplish this, the C-terminus of the Ub derivatives was either deleted (UbΔ) or significantly modified (Ubm), rendering the expressed Ub protein derivatives incapable of being activated and subsequently donating their C-terminus for isopeptide bond formation (Wilkinson and Audhya, 1981). The result of this modification to the Ub derivatives is that they must, by definition, exist within the cells as monomeric Ub with one exception (Figure 2.2). The only exception would be in the event that the available lysine(s) present is suitable as an acceptor for ubiquitination. In this instance, the Ub derivative would become covalently linked to a Ub monomer with an unmodified C-terminus, resulting in the formation of a Ub-Ub conjugate. The Ub monomers will also exist as the anchor or ultimate acceptor for any Ub chain formed. Thus, these Ub mutants will exist as conjugates of predictable molecular weights (18.5 kDa). If the C-terminus of the Ub derivatives was not mutated, then the Ub mutants could act as donors and be covalently bound to all natural Ub substrates *in vivo*, and be incorporated randomly into multi Ub chains at internal positions. Analysis of these proteins by SDS PAGE would give a variable range of weights which could not be predicted.

*Specific detection of mUb-UbΔ conjugates in whole cell lysates.*

Specific detection of Ub-Ub conjugates was facilitated by the introduction of an epitope-tagged donor moiety into this two plasmid system. For this experiment, the Ub donor was an N-terminal Myc-epitope tagged unmodified Ub derivative (denoted as mUb) which had previously been shown to allow for unambiguous detection of the untagged Ub-protein conjugates formed *in vivo* (Ellison and Hochstrasser, 1991). It had been observed that the *in vivo* expression of Ub that is N-terminally tagged with a peptide epitope results in the formation of tagged Ub -protein conjugates that are detectable by immunoblotting with a monoclonal antibody that recognizes the tag (Ellison and Hochstrasser, 1991) and, furthermore, were shown to form correct isopeptide bonds . The mUb-UbΔ conjugate is of a predictable size (18.5 kDa), and will be present only when a mUb-UbΔ linkage forms between a suitable lysine on the acceptor derivative and the C-terminus of the mUb donor. It is predicted that a UbΔ derivative with all ubiquitinatable lysines converted to arginines will be unable to form a mUb-UbΔ conjugate. Therefore, if in fact only one other lysine

than position 48 is acceptable of isopeptide bond formation, only one of the UbΔ acceptors should be incapable of forming the mUb-UbΔ conjugate. This would clearly define the precise alternative lysine residue effective for Ub-Ub linkage formation *in vivo*.

*Introduction and expression of Ub derivatives in vivo.*

The two plasmids required for the Ub conjugation assay were introduced into yeast by cotransformation. Expression of the Ub derivatives in *S. cerevisiae* was facilitated by the presence of the *CUP1* promoter which highly expressed both the mUb and UbΔ derivatives when grown in the presence of the divalent copper ion (Figure 2.1). The protein encoded by the UbΔ derivatives appeared as noticeable monomeric Ub proteins of approximately 8.5 kDa when stained with Coomassie blue (see Figure 3.5). The mUb protein, due to the additional sequences introduced by the Myc-epitope tag, migrates at a slightly greater molecular weight of approximately 9.5 kDa, a sufficient difference to distinguish between the two derivatives on an 18% acrylamide gel (see Figure 2.3 for example). Both the mUb and UbΔ derivatives are capable of being overexpressed and furthermore appear to be stable proteins *in vivo* , suggested by their ability to accumulate to high levels without being turned over.

*In vivo expression and immunoblot detection of Ub-Ub conjugates*

The individual plasmids encoding the UbΔ acceptor derivatives and the mUb donor derivative were cotransformed into *S. cerevisiae* and their expression was induced prior to whole cell lysis in preparation for SDS PAGE and immunoblot analysis (Appendix A). Overexpression of these Ub derivatives overcomes competition between the plasmid borne Ub derivatives and Ub that is normally expressed from *UBI1*, *UBI2* and *UBI3*, such that the mUb-UbΔ conjugates can be observed after immunoblot analysis.

Western analysis of total protein from yeast cells coexpressing the tagged Ub donor in combination with the untagged UbΔ acceptors is shown in Figure 2.3. The ability of mUb to form conjugates with a range of proteins substrates in addition to the UbΔ acceptor resulted in the complicated banding pattern spanning the entire molecular weight range of proteins (Lane m), however, the most prevalent bands are the mUb monomer (9.5 kDa), and the mUb-mUb dimer band (20 kDa), as indicated. With the exception of the no-Myc control lane (Lane -), these two tagged species are prevalent in all samples, as expected. Notably, those samples also expressing the UbΔ derivatives clearly have a prevalent band appearing slightly below the mUb-mUb band that is faint in the mUb-only sample. This faint band is also seen in the no-Myc control lane (Lane-) and is therefore due to antibody cross reactivity. Based on these controls we conclude that the prevalent band found in the

mUb-UbΔ lane is the mUb-UbΔ conjugate. This conjugate is present at the predicted molecular weight (approximately 18.5 kDa), which is slightly lower than the mUb-mUb dimer as a consequence of the absence of a second Myc-epitope. It can be seen that each of the doubly mutated UbΔ acceptor derivatives are capable of forming the mUb-UbΔ conjugate expressed in *S. cerevisiae*.

These results suggest two immediate possibilities. First, a site other than lysine may be utilized in Ub to form Ub-Ub conjugates. Secondly, it is possible that more than two lysine residues within Ub can be targeted for isopeptide bond formation by the C-terminus of these Ub donors. Experiments were therefore developed to address each of these possibilities.

### 2.3.2. R48Ub dimers are not linked via an amino terminal linkage.

The possibility existed that Ub may become covalently bound to another Ub protein through an amino acid residue other than lysine. Possibilities included the formation of an ester linkage with nucleophilic side chains including serine, threonine, or tyrosine residues, or alternatively by an isopeptide bond occurring at the α-amino group of the N-terminus of the accepting Ub monomer.

To address this problem, it was necessary to isolate a sufficient quantity of the UbΔR48-mUb conjugates for linkage analysis. Since it is clear from Figure 2.3 that mUb-UbΔR48 conjugates constitute a considerable fraction of the total conjugates formed, we developed a purification strategy based on the acid solubility of these species coupled with preparative PAGE (see Materials and Methods). Large volume yeast cultures coexpressing UbΔR48 (or UbΔ as a control) and mUb were grown in the presence of copper to induce the expression of the Ub derivatives *in vivo*.

*Isolation of Ub conjugates.*

Ub protein is extremely stable to a wide range of pH. We took advantage of this property to develop a means of specifically extracting Ub, mUb and Ub conjugates from whole cell lysates by acid extraction. The drastic drop in pH upon addition of acid to a cell lysate causes the majority of cellular proteins to denature and aggregate. Ub, however, which is considerably more stable to extreme conditions, was predicted to be more soluble under acidic conditions than the majority of cellular proteins. As seen in Figure 2.4, treatment of yeast lysates at pH 1.0 yielded an optimal solubility of UbΔ, mUb and the mUb-UbΔ conjugates relative to total yeast proteins. Although the mUb-UbΔ dimeric conjugate is not easily apparent in this figure, anti-Ub Western analysis of the acid soluble

and insoluble fractions demonstrated that the majority of Ub protein remained soluble after pH 1.0 acid treatment (data not shown).

Based on this information, acid extraction was used to partially purify [$^{14}$C]lysine labeled Ub monomeric and dimeric derivatives from yeast lysates. Additional purification of these species was carried out by SDS PAGE separation followed by electroblotting onto an Immobilon-P membrane. Monomeric and dimeric Ub derivatives were then excised and eluted from the membrane in preparation for Cyanogen Bromide (CNBr) cleavage (see Materials and Methods).

*Distinguishing between branched and linear conjugates by CNBr cleavage.*

A mUb-UbΔ dimer that links the C-terminus of one Ub monomer with the N-terminus of another via a peptide bond can be distinguished from a branched mUb-UbΔ dimer by CNBr cleavage at methionine residues. There are three methionine residues present in a mUb-UbΔ conjugate for both the linear and branched dimers. In both cases, one is at the N-terminus of the UbΔ protein, and one at the N-terminus of the Myc-tag. The third methionine is found immediately following the Myc tag, at position 1 of the Ub sequence (see Figure 2.5a). In a branched mUb-UbΔ dimer, the Ub moieties will be covalently bound at internal Ub residues. Whereas CNBr cleavage will result in the cleavage of the Myc tag from both dimer configurations generating products that are equivalent in size to a Ub-UbΔ dimer only the linear dimer will be cleaved to monomeric Ub owing to the presence of the single methionine situated at the Ub-UbΔ boundary. These two possibilities can be readily distinguished by SDS PAGE.

One half of each immobilized sample was reacted *in situ* with CNBr and then separated by SDS PAGE for analysis of the cleavage products. The autoradiogram of this gel is presented in Figure 2.5b. A comparison of monomeric K48UbΔ protein before CNBr exposure (Lane 3) and after (Lane 5) indicates that CNBr does not cleave within the Ub protein itself as expected. However, a comparison of mUb before CNBr treatment (Lane 4) and after CNBr exposure (Lane 6) clearly indicates that the Myc epitope is cleaved from the protein almost to completion. The isolated mUb-UbΔ dimer band is shown untreated (Lane 7) and after CNBr treatment (Lane 9), and it can be seen that increased mobility of the single major CNBr product corresponds well with the predicted molecular weight (16 kDa) of a Ub-UbΔ dimer that has lost the Myc tag. A similar observation was made for the untreated (Figure 2.5 Lane 8) and CNBr treated (Lane 10) mUb-UbΔR48 dimer, where there is a shift in molecular weight that corresponds to the formation of the Ub-UbΔ dimer. The faint lower molecular weight bands observed in these lanes are probably due to non-specific protein degradation. In neither case does monomeric Ub

appear at the predicted 8.5 kDa molecular weight position, strongly implying that in our system, the UbΔR48 conjugate is not linked through its amino terminus. As a final control, the proteins isolated originally and generated after CNBr cleavage were shown to contain Ub by anti-Ub Western analysis (data not shown)

### 2.3.3. Lysines at positions 29, 48 and 63 of Ubiquitin are sites for Ubiquitination.

To determine if the alternative sites for Ub-Ub conjugate formation occurred exclusively at lysine residues, a series of yeast plasmids was created, each expressing a Ub acceptor derivative (UbΔ) in which all but one lysine was replaced with arginines. Detection of these derivatives and their conjugates in yeast was facilitated by the presence of a Myc-epitope tagged Ub derivative (mUb) acting as the donor in the assay, as in previous experiments. It was anticipated that if lysine residues other than K48 functioned as alternative linkage sites, then UbΔ derivatives carrying single targetable lysines would form a detectable mUb-UbΔ conjugate with the predicted molecular weight of 18.5 kDa after anti-Myc immunoblot analysis. Alternatively, if other non-lysine sites functioned as conjugation sites, it was predicted that a UbΔ derivative lacking all lysines would still be capable of forming the mUb-UbΔ conjugate. After cotransformation and overexpression of the individual Ub acceptors harboring all (UbΔ.K+), none (UbΔ.$K_0$), or one lysine residue (UbΔ.K), it was determined that three Ub derivatives were capable of functioning as acceptors for ubiquitination (Figure 2.6). As before (Figure 2.3), the mUb and mUb-mUb dimer bands are prevalent after Western analysis for reasons previously explained. A wild-type UbΔ in conjunction with mUb (Lane +) shows the augmentation of a previously faint band at the position expected for a mUb-UbΔ conjugate. Three single lysine UbΔ derivatives presented this same mUb conjugate, specifically those derivatives carrying lysines at position 29, 48 and 63. This was demonstrated by the presence of the mUb-UbΔ conjugate in these lanes after Western analysis. In contrast, derivatives with either no lysines or single lysines situated at position other than 29, 48, or 63 failed to be ubiquitinated. The simplest conclusion that can be drawn from these results is that the K29UbΔ, K48UbΔ, and K63UbΔ derivatives are each ubiquitinated at K29, K48 and K63, respectively. Furthermore, these results suggest that it is specifically lysines that are targeted for ubiquitination and other amino acids are not being utilized for alternative Ub-Ub linkage formation based on the inability of the R7 UbΔ derivative to be ubiquitinated. Interestingly, it was also noted that the expression of UbΔ in combination with mUb relative to that of cells expressing mUb alone results in overall higher background band detection.

### 2.3.4. A Ub acceptor tagged at its C-terminus is also ubiquitinated at positions 29, 48 and 63.

It seemed possible to simplify the interpretation of the anti-Myc western analysis by reducing the background bands resulting from the ability of mUb to conjugate to a variety of cellular proteins *in vivo*. By transferring the Myc-epitope from the donor (mUb) to the C-terminus of the single lysine acceptor derivatives of Ub (denoted as Ubm), it was predicted that not only would the epitope tag block the C-terminus, but it would also result in Ubm being the only detectable protein in whole cell lysates by Western analysis with anti-Myc antibody.

The series of single lysine UbΔ derivatives was converted to Ubm derivatives by replacing the internal sequences of a Ubm gene cassette with the DNA sequences of the single lysine derivatives (Appendix B). The resulting proteins were thus expressed as fusions between a C-terminal Myc epitope tag, and Ub expressing none (Ubm.$K_0$), one (Ubm.K), or all (Ubm.K+) lysines. The Ub donor in this case is wild type, untagged Ub expressed from a high copy *URA3* based yeast plasmid. As before, the two plasmid expression system was used to determine, and confirm, the sites of alternative Ub-Ub linkage *in vivo*.

The SDS Western analysis of total protein from yeast cells overexpressing the tagged Ubm acceptors and the Ub donor plasmid is shown in Figure 2.7. Three of these Ubm derivatives formed Ub-Ubm conjugates when expressed in *S. cerevisiae:* Ubm.K29, Ubm.K48 and Ubm.K63. As expected, derivatives with either no lysines (Lane 0), or lysines situated at positions other than 29,48 and 63 failed to be ubiquitinated. Unlike the UbΔ derivatives (Figure 2.6), the banding pattern for Ubm derivatives detected with the Myc antibody is greatly simplified and clearly demonstrates how the Ub conjugates are restricted to position 29, 48 and 63. Therefore, the addition of a Myc epitope tag to the single lysine Ub derivatives does not effect ubiquitination, but in fact reaffirms that positions 29, 48 and 63 are targets for ubiquitination.

### 2.3.5. Targeting of Ub to K29, K48 and K63 in Ub results from recognition of native Ub structure.

In view of the generally accepted role of the Ub system in the turnover of damaged proteins, it was possible that the ubiquitination of lysines at positions 29, 48 and 63, whether in a UbΔ or Ubm context, resulted from the recognition of structural damage that may have been introduced by the various modifications made to the Ub polypeptide sequence. The mutational load of up to 9 amino acids altered out of 76 (i.e. UbΔ.$K_0$) may well perturb the structure of the Ub protein despite the conservative replacement of lysine

with arginine. In addition, the Ubm derivatives also have the 10 amino acid long Myc-tag. To test this possibility, we assessed the effect of the mutations present in the Ub derivatives by testing the ability of a heavily mutated Ub derivative to act as a donor for ubiquitination. To behave as a Ub donor, the C-terminus of the Ub protein must be activated. Activation requires that the Ub protein not only be recognized and activated by the Ub activating enzyme E1, but also that it then be transferred to an E2 and ultimately be linked to a second Ub protein to form a Ub-Ub conjugate.

We examined the effect of multiple lysine substitutions on other Ub related function by testing whether or not a Ub derivative carrying arginine mutations at all seven lysines positions, and an N-terminal Myc-tagged (mUb.$K_0$), could function as a donor in Ub-Ub conjugation. The intact C-terminus of this derivative allowed the protein to act as a Ub donor. SDS-Western detection using anti-Myc will detect monomeric mUb.$K_0$ and the mUb.$K_0$-Ub conjugate formed if mUb.$K_0$ is activated and targeted. The overexpression of wild-type Ub was included to provide consistent levels of Ub acceptors as compared to Figures 2.1, 2.4 and 2.5. Western analysis of total protein from yeast cells coexpressing mUb.$K_0$ and untagged wild-type Ub (Figure 2.8) clearly shows that a mUb.$K_0$-Ub conjugate is being formed *in vivo*. Furthermore, the mUb.$K_0$ dimer levels are similar to other conjugate levels such as mUb-mUb dimer levels (Figure 2.6, Lane C), relative to their respective monomeric forms. The result of this experiment clearly demonstrates that the mUb.$K_0$ protein can be recognized and targeted by the other protein components of the Ub proteolytic pathway, strongly suggesting that there is no obvious conjugation defect associated with complete lysine replacement nor the presence of the Myc-epitope tag and that, therefore, mUb.$K_0$ maintains its native form.

On the basis of the above observations, we concluded that the targeting of K29, K48 and K63 resulted from recognition of a native Ub structure. However, knowing that only three lysines were used for conjugate formation allowed us to reduce the mutational load by altering only the lysines at positions 29, 48 and 63. It may be that the mutation of all seven lysines (Ubm.$K_0$) in some manner prevented a fourth ubiquitination site from being detected. To test the validity of this hypothesis, a series of Ubm derivatives were created which carried two or three lysine-to-arginine conversions at positions 29, 48 and 63 (Appendix B). These Ubm acceptor plasmids were cotransformed with a second plasmid expressing wild type Ub as a donor, and analyzed by SDS Western blotting.

Western analysis of total protein from yeast cells coexpressing these Ubm derivatives and untagged Ub is shown in Figure 2.9. As expected, a derivative carrying substitutions at all three positions failed to serve as a substrate for conjugation (Lane 0), whereas conjugation was restored upon reintroduction of a single lysine at any of the three

positions (remaining lanes). Thus, the results from this limited substitution experiment paralleled the results of Figures 2.4 and 2.5.

## 2.4 Discussion

While there is considerable evidence that Ub dependent proteolysis is facilitated by the assembly of K48 multiUb chains onto the targeted substrate (Hochstrasser *et al.*, 1991; Chau *et al.*, 1989; Gregori *et al.*, 1990; Finley *et al.*, 1994), the possibility of other types of chain linkages has been recognized *in vitro* (Haas *et al.*, 1991; Gregori *et al.*, 1990; Hadari *et al.*, 1992 ) and *in vivo* (Johnson *et al.*, 1992). In no instance, however, have these linkages been characterized with respect to structure or function. To examine the question of alternative chain linkages further, we designed a series of Ub expression plasmids which, when expressed in yeast in appropriate combinations, facilitated the detection of multiUb chains *in vivo* and the manner by which the Ub molecules were linked. Using this yeast based system, we determined that in addition to the well characterized K48 Ub-Ub linkage (Chau *et al.*, 1989), Ub could also be linked to itself by isopeptide bond formation at lysine 29 and lysine 63.

One possible explanation for these findings is that the various modifications made to the Ub polypeptide produces structural perturbations which in turn result in the recognition of these derivatives by the Ub proteolytic system as damaged substrates. In this scenario, Ub derivatives would themselves be ubiquitinated and degraded. We have discounted this possibility for a number of reasons. First, all of the lysine-to-arginine substitutions occur at positions on the surface of the Ub molecule. In view of the conservative nature of these replacements, structural changes are expected to be minimal or nonexistent. Secondly, fully substituted Ub functions both as an E1 and E2 substrate (Figure 2.8). Finally, highly substituted Ub is as resistant to trypsin cleavage as unmodified Ub (Appendix C, Figure C.5), providing strong evidence that its 3D structure is uncompromised. Based on these findings and the results presented in Chapter 3, we have concluded that the ubiquitination of Ub at positions 29 and 63 are biologically relevant modifications and may represent distinct signals for other Ub-dependent processes.

Whereas the K48 chains that are targeted to many substrates constitute a spectrum of chains species extending from one to many Ub moieties in length, our derivatives terminate predominantly at the Ub-Ub dimer, regardless of linkage type. The biochemical basis of this accumulation is unclear, but may reflect the fact that the derivatives that anchor the chain in our situation are not substrates for Ub-dependent proteolysis (results not shown).

From a structural perspective, the lysines at positions 29, 48 and 63 are separated from one another by a considerable distance on the surface of the Ub molecule (Figure 2.10, from Vijay-Kumar *et al.*, 1987). Therefore, the structure of Ub chains composed of different linkages will vary from one another markedly. A sense of the structural complexity that becomes possible with these three coupling positions is illustrated in Figure 2.11. The most complex configurations naturally arise from the combination of different linkages within the same chains, or from the possibility that one Ub molecule can serve as a branch point for several linkages. While our experiments have not addressed these possibilities, the observed and potential variations in chains configuration, combined with the notion that different configuration perform different functions, highlight the versatility of Ub as a postranslational signal. In Chapter 3, we present evidence that one of these configurations plays an important role in the yeast stress response.

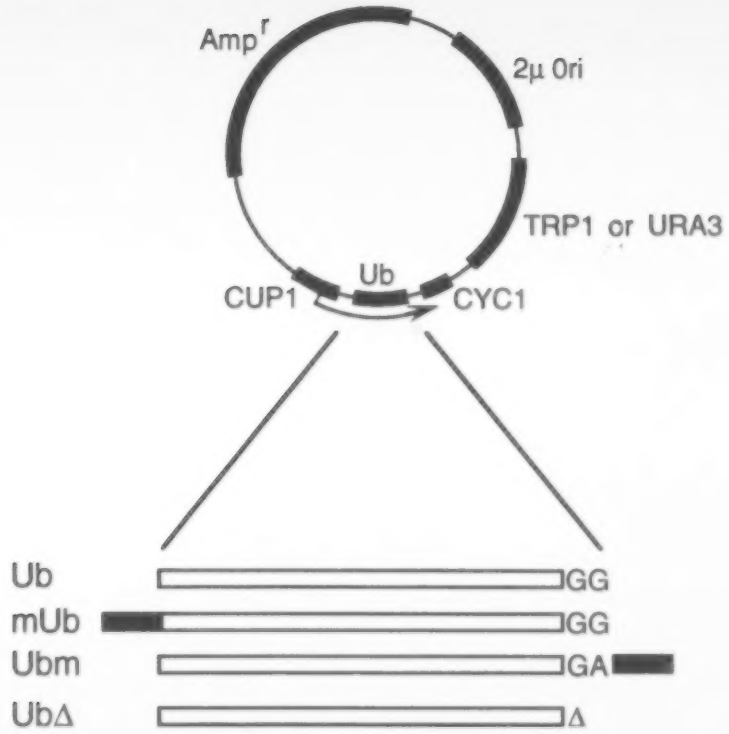



FIGURE 2.1

**Yeast Expression Vectors and Ub cassette derivatives**

A plasmid map indicating relevant regions of the yeast parental vectors used in the construction of the mUb, Ubm, and UbΔ derivatives is shown. The parental yeast vectors used in these experiments contain similar components. These included the presence of the ampicillin (Amp$^r$) marker for plasmid maintenance in *E. coli* ( a yeast shuttle vector), and either the *TRP1* or *URA3* marker for selection in *S. cerevisiae*. Abbreviations are as follows: *CUP1*, yeast copper metallothionein promoter; *CYC1*, *CYC1* transcriptional terminator; *TRP1*, 5' P-ribosyl-anthranilate isomerase gene; *URA3*, orotidine-5'-P-decarboxylase gene; 2 μ Ori, a portion of the 2 μ plasmid necessary for high copy number; mUb, wild type Ub gene cassette with an N-terminal fusion with the DNA sequence encoding for the 10 amino acid Myc epitope sequence; Ubm, wild type Ub gene cassette with a C-terminal fusion to the sequences encoding for the Myc epitope; UbΔ, wild type Ub gene cassette deleted for the final two amino acids, glycine75 and glycine76.

FIGURE 2.2

**Ub donor/Ub acceptor Schematic.**

I. A generic Ub-Ub conjugate is shown. The C-terminus of each Ub moiety is represented as a rectangle (i), and the remainder of the protein, including the seven lysine (K) residues (iii) are represented as an oval. The acceptor is the Ub protein whose lysine residue(s) is used to create the isopeptide linkage (ii) with the intact and available C-terminus of the Ub donor moiety.

II. *A summary of the four Ub derivatives used.* mUb, unlike Ub, can be detected by Western analysis using the Myc antibody, due to the N-terminal Myc-epitope tag. This tag increases the molecular weight of mUb sufficiently that the difference can be detected by SDS PAGE. Both Ub and mUb have intact C-termini and can, when suitable lysines are present, act as both a donor and acceptor in conjugate formation. The Ub-Ub conjugate is not detectable by anti Myc Western analysis, but the mUb-mUb dimers are detectable after SDS PAGE and Western analysis as ~20 kDa conjugates.

The Ubm derivative carries the Myc epitope and is the same size as mUb, but the tag is present at the C-terminus. This tag blocks the C-terminus, preventing Ubm from acting as a donor in conjugate formation. Similarly, the UbΔ derivative acts only as an acceptor due to the two amino acid deletion (Gly75 Gly76) at the C-terminus. The UbΔ derivative does not express the Myc epitope, is not detectable by Western analysis, and is the same size as wild type Ub.

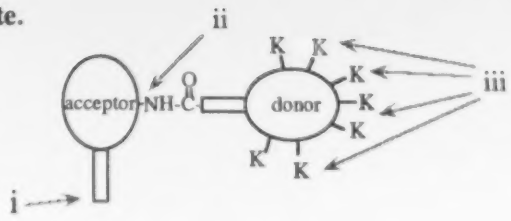
III. a. The coexpression of UbΔ with mUb *in vivo* will result in the formation of mUb-UbΔ conjugates of a predictable molecular weight complex of 18.5 kDa after SDS PAGE, distinct from the ~20 kDa mUb-mUb conjugate which can also form (see above). As indicated, the UbΔ derivative must act as the acceptor, or anchor, in any conjugates formed. Expression *in vivo* also allows for wild type, endogenous Ub to be incorporated into these conjugates. An example of this combination is shown by the trimer formation.

b. When UbΔ derivatives are mutated at lysine residues, they will act as acceptors only if the lysine(s) remaining are suitable for conjugate formation. If acceptable, a portion of the UbΔ derivatives produced will form 18.5 kDa mUb-UbΔ conjugates that are detectable by anti Myc Western analysis. If unacceptable, this conjugate will not be detected.

IV. a. The expression of Ubm *in vivo* will result in the detection of monomeric (9.5 kDa) and the 18.5 kDa Ub-Ubm dimer when a conjugate is formed. Wild type Ub, expressed from a second plasmid, and/or endogenous Ub, acts as the donor moiety.

b. As for UbΔ, when the Ubm derivative is mutated at lysine residues, it will act as an acceptor only if the lysine(s) remaining are suitable for conjugation. If acceptable, a portion of the Ubm derivatives produced will form 18.5 kDa conjugates that are detectable by anti Myc Western analysis. If unacceptable, monomeric Ubm will be detected in the absence of Ub-Ubm conjugates.

**I. The Ub-Ub conjugate.**

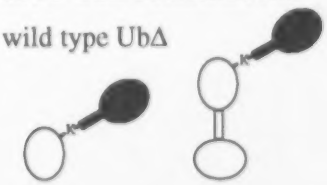



**II. Wild type Ub, mUb, Ubm, and UbΔ**

| Ub | mUb | UbΔ | Ubm | derivative |
|---|---|---|---|---|
| ~8.5 kDa | ~9.5 kDa | ~8.5 kDa | ~9.5 kDa | size |
| both | both | acceptor | acceptor | donor/acceptor |
| no | yes | no | yes | antiMyc detection. |

**III. The mUb-UbΔ combination.**

a. wild type UbΔ



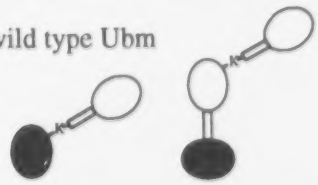
| dimer | trimer |
|---|---|
| 18.5 kDa | ~27 kDa |

b. Lysine-to-arginine UbΔ derivative

| unsuitable lysines | acceptable |
|---|---|
| no conjugate | 18.5 kDa |

**IV. The Ub-Ubm combination**

a. wild type Ubm



| dimer | trimer |
|---|---|
| 18.5 kDa | ~27 kDa |

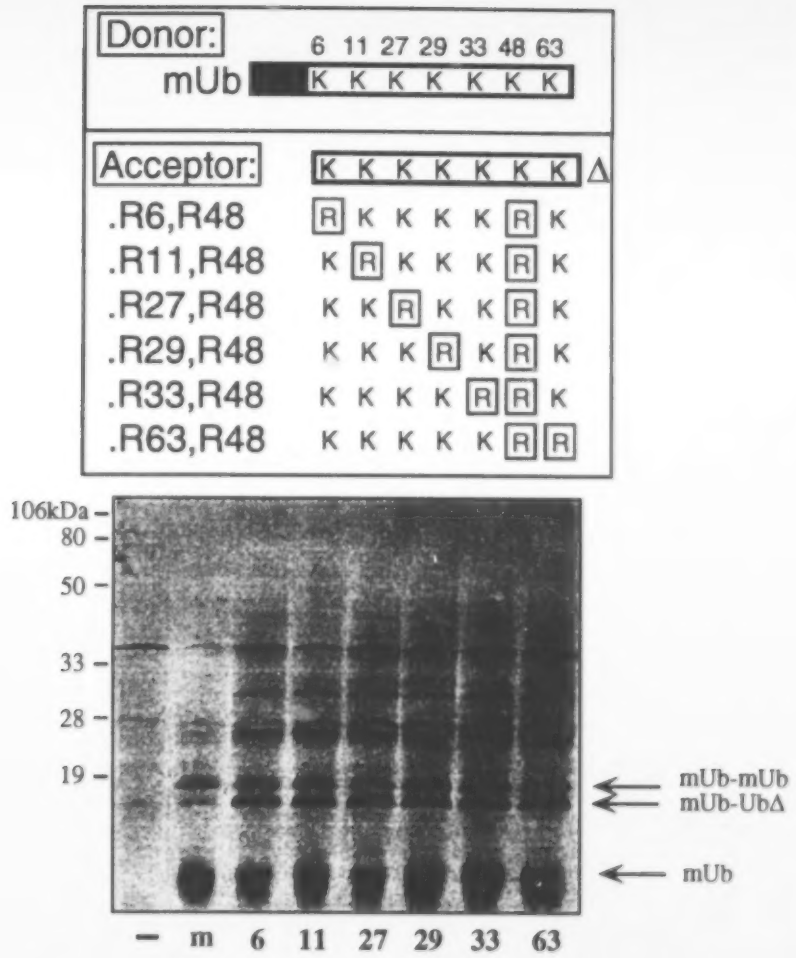
b. lysine-to-arginine Ubm derivative

| unsuitable lysines | acceptable |
|---|---|
| ~9.5 kDa | 18.5 kDa |

FIGURE 2.3

**Multiple ubiquitination sites within Ub.**

Shown is an SDS-PAGE Western blot of total protein from yeast cells coexpressing an N-terminal Myc-epitope tagged Ub protein (mUb) in combination with untagged Ub derivatives with Gly75 and Gly76 deleted (UbΔ) and probed with anti-Myc antibody. This Western was developed using the Alkaline Phosphatase color reaction (Appendix A). The schematic indicates the pairs of Ub derivatives expressed in each lane. Donor, the Ub moiety that contributes its C-terminus to conjugate formation, present on a *URA3* yeast vector. Acceptor, the targeted UbΔ derivative present on a *TRP1* yeast vector. Numbers mark the position of the seven lysines present in the wild type Ub sequence. Lysine to arginine replacements for each Ub derivative have been boxed. The Myc-epitope tag is shown as a black box. For the gel, the position of monomeric (mUb), and conjugated (mUb-UbΔ) Ub derivatives are indicated. Gel lane numbers indicate the lysine, in addition to position 48, that have been converted to arginine for each UbΔ acceptor. Lane - ; yeast cells coexpressing two Ub derivatives, neither of which have a Myc epitope. Lane m; wild type untagged Ub coexpressed with mUb.


Donor:
6 11 27 29 33 48 63
mUb K K K K K K K
Acceptor:
K K K K K K K Δ
.R6,R48 R K K K K R K
.R11,R48 K R K K K R K
.R27,R48 K K R K K R K
.R29,R48 K K K R K R K
.R33,R48 K K K K R R K
.R63,R48 K K K K K R R
106kDa
80
50
33
28
19
mUb-mUb
mUb-UbΔ
mUb
– m 6 11 27 29 33 63

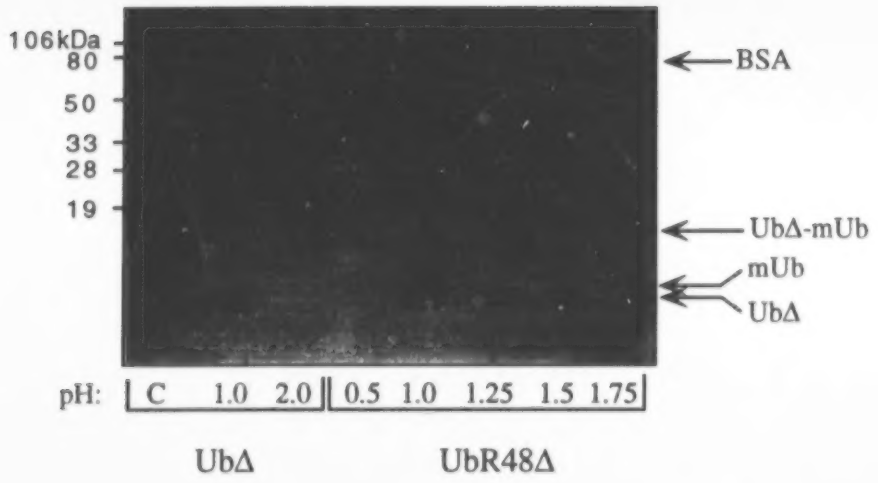



FIGURE 2.4

**Partial purification of Ub derivatives at low pH.**

Shown is a Coomassie stained 18% acrylamide gel of acid soluble protein derived from whole cell lysates of yeast (SUB60) expressing mycUb (mUb) in combination with UbΔ (UbΔ lanes) or mUb in combination with UbR48Δ (UbR48Δ lanes). The pH of extracts prior to sample concentration and electrophoresis is indicated. Also indicated are the electrophoretic positions of bovine serum albumin (BSA) which was included as a carrier, the mUb-UbΔ conjugate and molecular weight standards. Lane C is an untreated control.

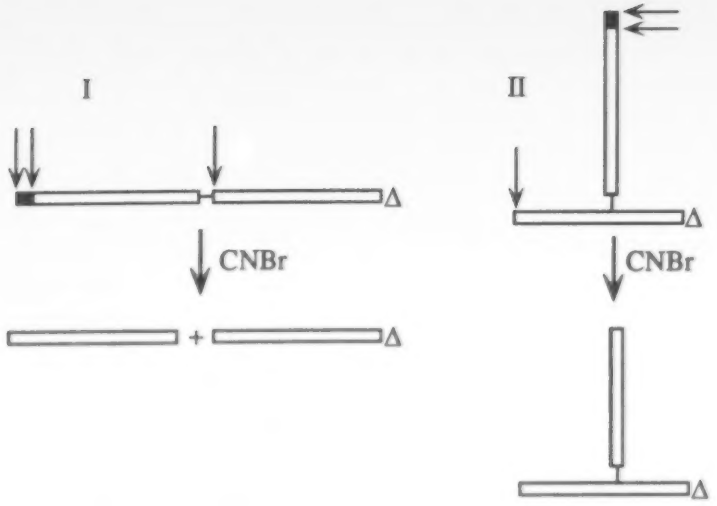
FIGURE 2.5

**Ubiquitin Dimers are not linked through an α-amino peptide bond.**

Shown in Part A is a schematic representation of the strategy used to distinguish between an N-terminally linked Ub-Ub conjugate and a branched Ub-Ub conjugate by cyanogen bromide (CNBr) treatment. Shown in Part B is an autoradiogram of the products of CNBr cleavage separated on an 18% SDS PAGE gel. [$^{14}$C]lysine labeled MycUb (mUb), UbΔ, UbR48Δ, mUb-UbΔ dimer and mUb-UbR48Δ dimer were purified and treated CNBr as described (Materials and Methods).

Lanes: Total protein from yeast expressing mUb in combination with either UbΔ (Lane 1) or UbR48Δ (Lane 2), purified UbΔ (Lanes 3 and 5), mUb (Lanes 4 and 6), mUb-UbΔ (Lanes 7 and 9), mUb-UbR48Δ (Lanes 8 and 10). Also indicated is the position of the Ub-UbΔ dimer resulting from CNBr cleavage.

A.


I
II
Δ
CNBr
+
Δ
Δ
CNBr
Δ

B.

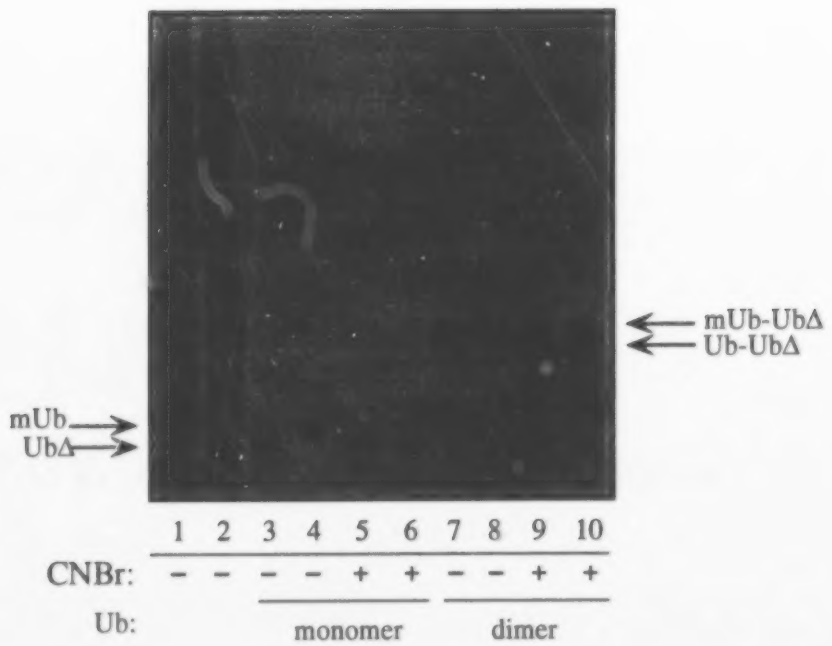

mUb-UbΔ
Ub-UbΔ
mUb
UbΔ
1 2 3 4 5 6 7 8 9 10
CNBr: – – – – + + – – + +
Ub: monomer dimer

FIGURE 2.6

**UbΔ derivatives with a single lysine at position 29, 48 or 63 can be targeted for ubiquitination.**

Shown is an SDS PAGE Western blot of total protein from yeast cells coexpressing N-terminal Myc epitope tagged unmodified Ub (mUb) and untagged Ub derivatives deleted for the C-terminal glycines (UbΔ) and probed with the anti Myc antibody. The UbΔ derivatives were altered to contain only one of the seven lysines normally present in Ub. Schematics indicate the pairs of Ub derivatives expressed in each experiment. Donor: the Ub moiety that donates its C-terminus to conjugate formation, in this case mUb. Acceptor: the targeted derivative that becomes ubiquitinated, in this case the series of UbΔ derivatives. Numbers mark the positions of the lysines (K) present on the wild type sequence (UbΔ.K+). Lysine to arginine (R) replacements for each derivative are boxed. Lysines empirically determined to be ubiquitinated are circled. The Myc epitope is displayed as a black box. For gels, the position of monomeric, dimeric, and trimeric Ub conjugates are as indicated. Gel lane numbers indicate the position of the single remaining lysine in each UbΔ acceptor. Control lane (Lane C) is mUb expressed without its UbΔ counterpart. Lane + is a UbΔ derivative with all lysines present (UbΔ.K+) coexpressed with mUb. Lane 0; UbΔ.$K_0$, (all lysines absent) coexpressed with mUb.

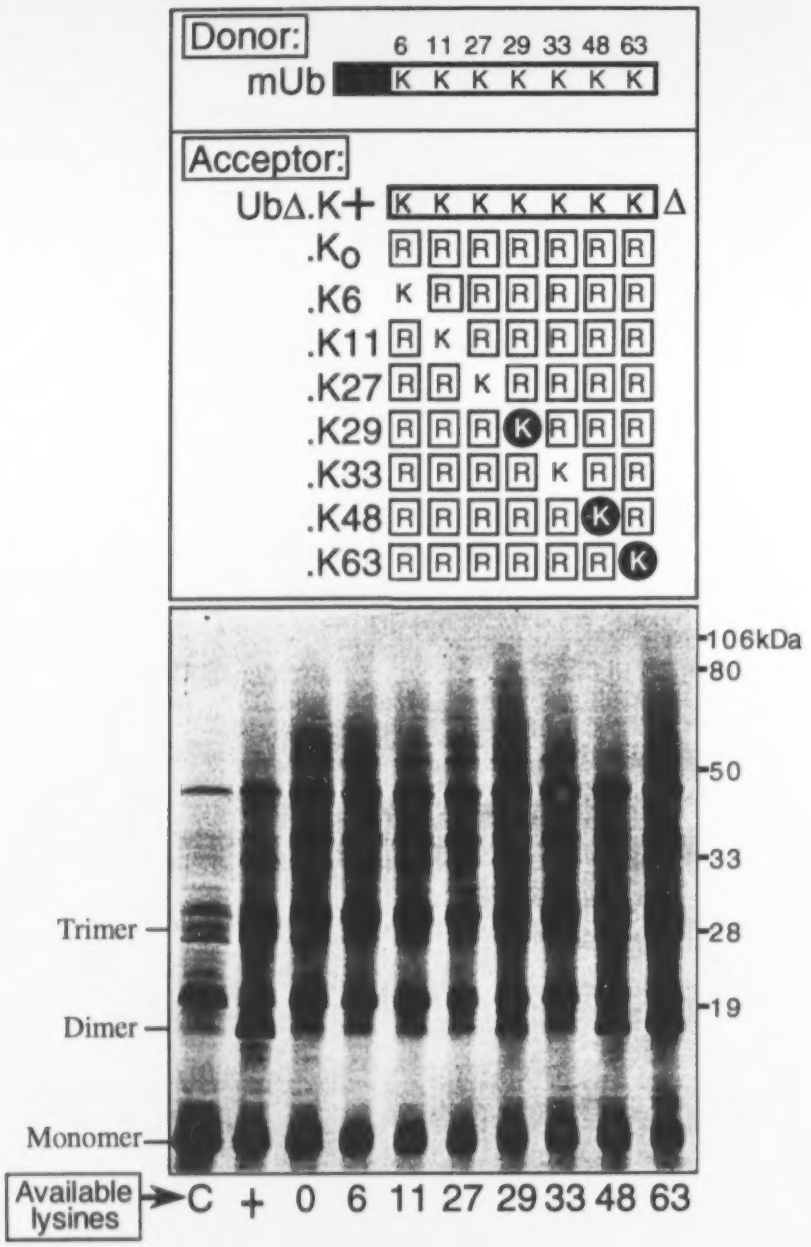
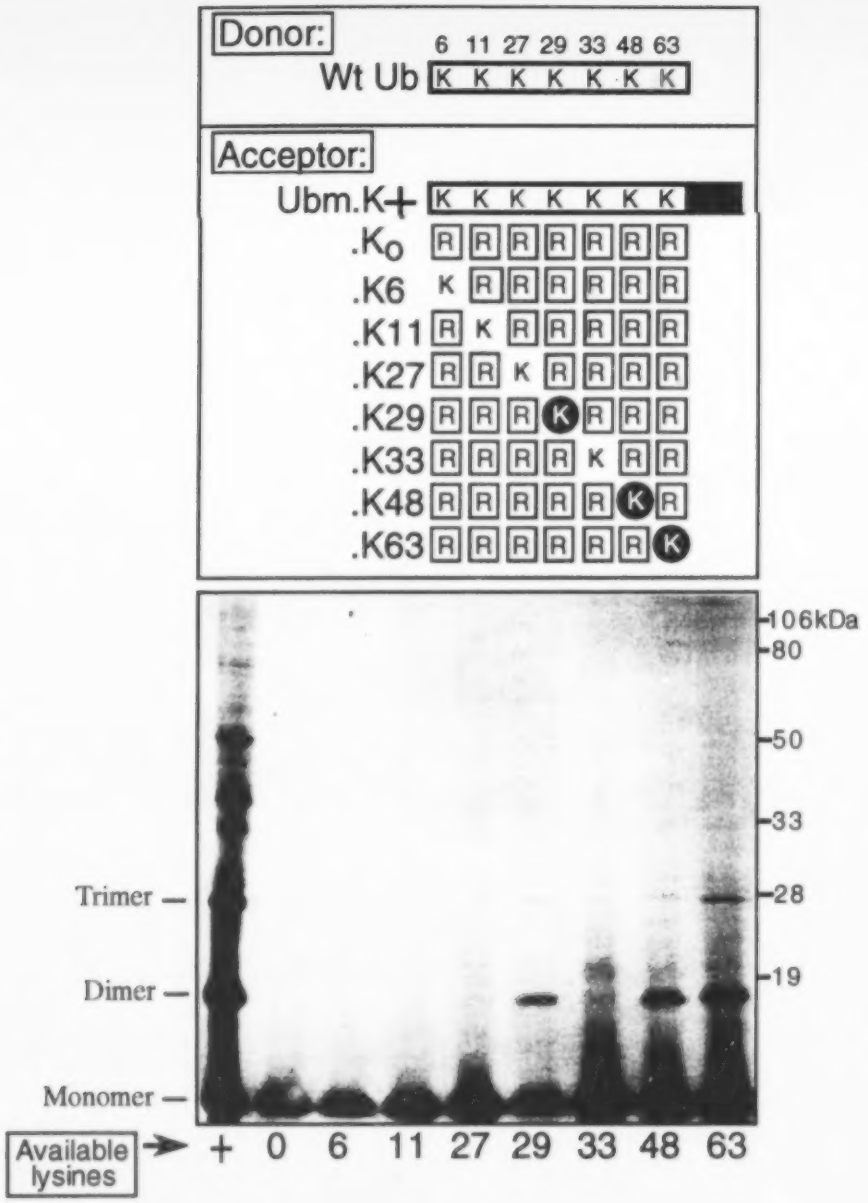

Donor:
6 11 27 29 33 48 63
mUb K K K K K K K
Acceptor:
UbΔ.K+ K K K K K K K Δ
.K0 R R R R R R R
.K6 K R R R R R R
.K11 R K R R R R R
.K27 R R K R R R R
.K29 R R R K R R R
.K33 R R R R K R R
.K48 R R R R R K R
.K63 R R R R R R K
106kDa
80
50
33
28
19
Trimer
Dimer
Monomer
Available lysines
C + 0 6 11 27 29 33 48 63

FIGURE 2.7

**Ubm derivatives expressing a single lysine at position 29, 48 and 63 are targeted for ubiquitination.**

Shown is an SDS PAGE Western blot of total protein from yeast cells coexpressing wild type Ub with C-terminally Myc-tagged Ub (Ubm). Ubm derivatives have been mutated to contain a single lysine at one of the seven lysine positions in Ub. The Western analysis was performed with the anti Myc antibody. Schematics indicate the pairs of Ub derivatives used in each experiment. Donor; the Ub moiety which donates its C-terminus to isopeptide bond formation in this instance wild type Ub expressed from a *URA3* yeast plasmid. Acceptor; the Ub moiety which becomes ubiquitinated, in this case the Ubm derivatives expressed from a *TRP1* vector. Numbers mark the position of lysines (K) present in the wild type Ub sequence (Ubm.K+). Lysine to arginine replacements for each derivative are boxed. Lysines empirically determined to be sites for ubiquitination are circled. The Myc epitope is shown as a black box. For gels, the position of monomeric, dimeric, and trimeric Ubm and Ubm conjugates are as indicated. Gel lane numbers indicate the single remaining lysine position of each acceptor. Lane +, Ubm.K+, all lysines present. O, Ubm.$K_0$, all lysines absent.


Donor:
6 11 27 29 33 48 63
Wt Ub K K K K K K K
Acceptor:
Ubm.K+ K K K K K K K
.K0 R R R R R R R
.K6 K R R R R R R
.K11 R K R R R R R
.K27 R R K R R R R
.K29 R R R K R R R
.K33 R R R R K R R
.K48 R R R R R K R
.K63 R R R R R R K
106kDa
80
50
33
28
19
Trimer
Dimer
Monomer
Available lysines
+ 0 6 11 27 29 33 48 63

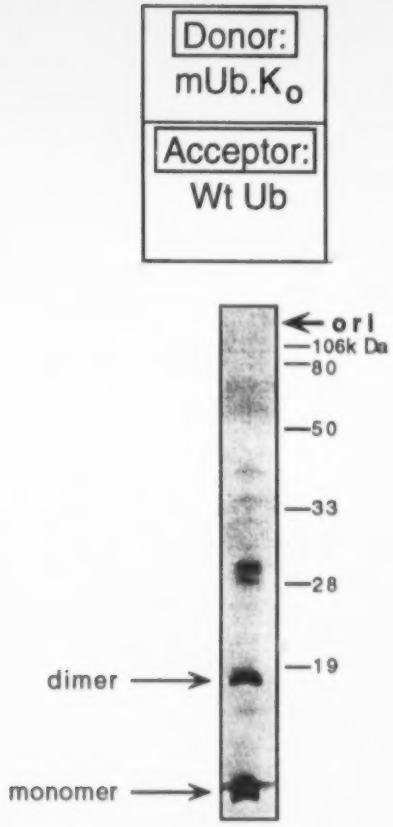



FIGURE 2.8

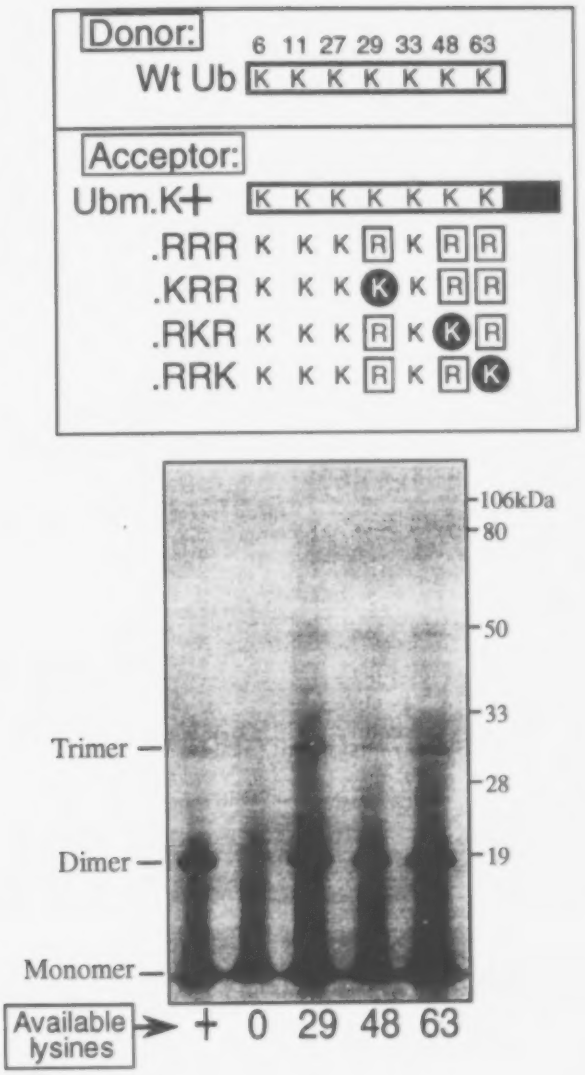
**A significantly mutated derivative of Ub can be recognized and activated by Ub system enzymes**

Shown is an SDS anti Myc Western blot of total protein from yeast cells coexpressing wild type Ub and an N-terminal Myc-tagged Ub derivative within which all lysines (K) have been substituted with arginines (mUb.Ko). Donor: the Ub moiety that contributes its C-terminus in the formation of a Ub conjugate. Acceptor: the targeted derivative in Ub conjugate formation. On the gel, the position of the monomer and dimer Ub moieties are indicated by arrows. Molecular weight standards are as indicated.

FIGURE 2 9

**A Ubm derivative with arginine mutations at positions 29, 48 and 63 is not targeted for ubiquitination.**

Shown is an SDS Western blot of total protein from yeast cells coexpressing wild type Ub with C-terminally Myc-tagged Ub (Ubm) and probed with the Myc antibody. For Ubm derivatives, only lysines identified to be targets of ubiquitination (circled positions) have been mutated to arginine in the combinations shown (boxed positions). Schematics indicate the pairs of Ub derivatives used in each experiment. Donor; the Ub moiety which donates its C-terminus to isopeptide bond formation in this instance wild type Ub expressed from a *URA3* yeast plasmid. Acceptor; the Ub moiety which becomes ubiquitinated, in this case the Ubm derivatives expressed from a *TRP1* vector. Numbers mark the position of lysines (K) present in the wild type Ub sequence (Ubm.K+). The Myc epitope is shown as a black box. For gels, the position of monomeric, dimeric, and trimeric Ubm and Ubm conjugates are as indicated. Gel lane numbers indicate the lysine position of each acceptor. Lane + (Ubm.K+), all lysines present. Lane O, (RRR.Ubm), linkage defective Ub acceptors with arginine mutations at positions 29, 48 and 63. Lane 29, a Ubm derivative expressing all lysines with two arginine mutations at positions 48 and 63. In a similar manner, lanes 48 and 63 refer to Ubm derivatives expressing only the indicated lysine of the three previously determined available for ubiquitination.


Donor:
6 11 27 29 33 48 63
Wt Ub K K K K K K K
Acceptor:
Ubm.K+ K K K K K K K
.RRR K K K R K R R
.KRR K K K K K R R
.RKR K K K R K K R
.RRK K K K R K R K
106kDa
80
50
33
28
19
Trimer
Dimer
Monomer
Available lysines
+ 0 29 48 63

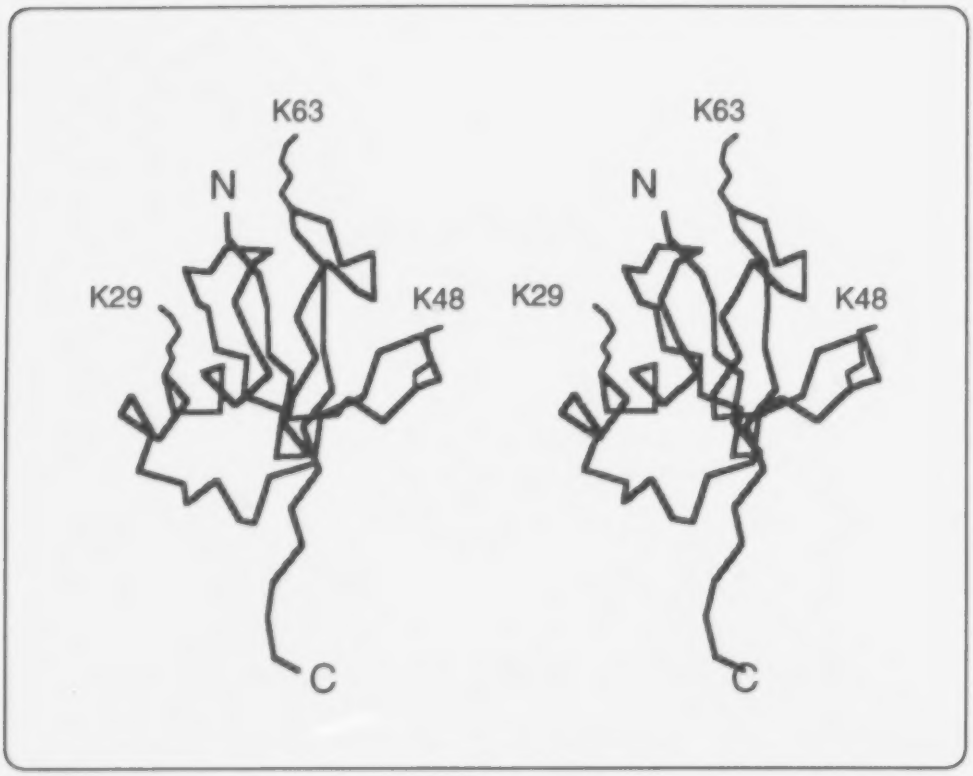



FIGURE 2.10

**3D stereo image of the Ub protein.**

3D stereo image of Ub showing lysines (K) targeted for ubiquitination (K29, K48 and K63). The amino terminus (N) and carboxyl terminus (C) are indicated. This figure was constructed using the Power MacImdad Interactive Molecular Display and Design software. Structural information is based on work by Vijay-Kumar et al., 1987.

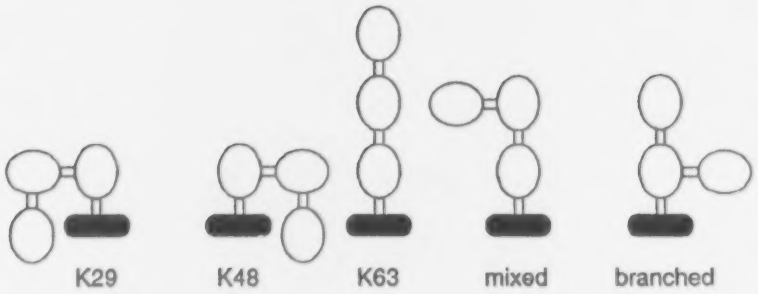



FIGURE 2.11

**Possible structures of alternative Ub-Ub linkages.**

Shown is a schematic of possible multiUb chain configurations based on the lysine geometry shown in Figure 2.10. Black ovals represent a nonspecific target protein.

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# CHAPTER 3. The Role of Alternative Ub linkages in the Yeast Stress Response.

## 3.1 Introduction

As described in Chapter 2, we have found that Ub monomers can be conjugated to one another *in vivo* through two novel linkage positions at lysine residues 29 and 63 of Ub. These alternative linkages, as well as the canonical K48 Ub-Ub linkage, are likely to be structurally different, as each of the three residues is found on opposing faces of the Ub protein (Figure 2.11). We therefore proposed that the Ub chain conformations produced by the formation of the three Ub-Ub linkages may act as different cellular signals and be used for different functions in the cell.

In addition to its documented role in degradation, there is evidence to suggest that ubiquitination can serve other functions in the cell. First, some ubiquitinated proteins are stable. Examples include the monoubiquitination of a histone protein (Pickart and Vella, 1988) and the immunoglobulin E receptor protein (Paolini *et al.*, 1993). Secondly, a Ub mutant unable to create the K48 Ub-Ub conjugate is still capable of complementing for the heat and amino acid analog sensitivities associated with the loss of the polyUb gene, *UBI4* (Finley *et al.*, 1994). This, therefore, suggests that Ub is functioning in the stress response in a manner distinct from that of the K48 proteolytic signal.

In this chapter, the role of the alternative Ub-Ub linkages *in vivo* was investigated. To begin, the Ub conjugating enzymes responsible for the formation of the novel linkages were identified. Since the individual Ub conjugating enzymes are involved in distinct processes in the cell, this would provide clues as to the role of the novel Ub-Ub linkages. Leading from this investigation, several functions were tested to determine the individual roles of each linkage type in the yeast stress response, in bulk and abnormal protein degradation, as well as in DNA repair. The results demonstrate that a novel Ub linkage is required for stress resistance in *S. cerevisiae* but not for general protein degradation, which has been previously demonstrated to be a function of the K48 multiUb chain. These investigations, therefore, provide evidence that the Ub-Ub linkages may function as discrete intracellular signals.

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A version of this chapter has been published. Arnason, T., and Ellison, M. (1994). Molecular and Cellular Biology. *14*, 7876-7883.

## 3.2. Materials and Methods

### *S. cerevisiae* Strains.

| Strain | Genotype | Source |
|---|---|---|
| SUB60 | *MATa his3-Δ200 leu2,-3,112 ura3-52 lys2-801 trp1-1 ubi4-Δ2::LEU2* | i |
| MHY508 | *MATα ubc4-Δ1::HIS3 ubc5-Δ1::LEU2 his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1* | ii |
| MHY501 | *MATα his3-Δ200 leu2-3,112 ura3-52 lys2-52 lys2-801 trp1-1 gal2* | ii |
| MHY497 | *MATα ubc1-Δ1::HIS3 his3-Δ200 leu2-3,112 ura3-522 lys2-801 trp1-1 gal2* | ii |
| MHY601 | *MATα ubc8-Δ1::URA3 ade2-1 ura3-1 his3-1 leu2-3,112 trp1-1 can1-100* | ii |
| KMY15 | *MATa ade2-1 his3-832 trp1-289 ura3-52* | iii |
| YKE41 | *MATa ade2-1 his3-832 trp1-289 ura3-52 rad6::ura3*** | iii |
| JP34G2 | *Matα ade2-1 his3Δ leu2Δ1 trp1 ura3-52 cdc34-2* | iv |

i. The yeast strain, SUB60 (*ubi4Δ*), a gift from D. Finley (Harvard) was used for the *in vivo* investigations to determine the role of the three Ub-Ub linkages in the yeast stress response and in proteolysis. ii. MHY508 (*ubc4/5Δ*), MHY501 (wild type), MHY497 (*ubc1Δ*), and MHY601(*ubc8Δ*) were gifts from M. Hochstrasser. iii. YKE41 is a *UBC2 (rad6)* deletion strain identical to KMY20 (genotype, *Mat* a, *ade2-1, his3-832, trp1-289, ura3-52, rad6Δ::URA3*; a gift from K. Madura) * except that a *ura3*$^-$ revertant was selected by FOA to allow for both *URA3* and *TRP1* based vectors to be maintained. KMY15 is a wild type strain isogenic to the YKE41 *rad6*$^-$ strain. iv. Yeast strain JP34G2 contains a temperature-sensitive *CDC34* (*UBC3*) allele (34°C nonpermissive temperature) and was a gift from J. Prendergast. These strains were used in the investigations to identify the Ub-conjugating enzymes responsible for the formation of the alternative Ub-Ub linkages.

### Testing E2 involvement in alternative Ub-Ub conjugate formation

Plasmid overexpression, sample preparation, and anti Myc Western analysis were carried out exactly as described in Appendix A, except that different yeast strains were used, and all Western development was done by ECL. Lastly, during induction, all strains were grown at 30°C with JP34G2 being the exception. Being a temperature sensitive strain

for CDC34 function, alternative linkage formation determination was done when the culture was grown at 32°C, slightly below the arrest temperature of 34°C (Goebl *et al.*, 1988).

**Quantitation of Myc-tagged Ubiquitin (Ub) and its conjugates from X-Ray film.**

The quantitation of Myc-tagged Ub for the purpose of determining the relative amounts of Ubm monomer and Ubm-Ub dimer was performed by selecting film exposures from ECL Western analysis that fell within the linear range of film density, followed by the densitometric scanning of appropriate bands, using a Joyce-Loebl Chromoscan 3 scanning densitometer.

**Phenotype Analysis.**

**a. Canavanine sensitivity analysis**

Cell survival in the presence of canavanine was determined by plating exponentially growing yeast cells (previously induced for 2 generations with $CuSO_4$ [100 μM]) on minimal media plates supplemented with histidine (10 mg/liter), lysine (40 mg/liter) and uracil (2.4 mg/liter) and also containing 1.7 μg canavanine per ml. The Ub derivative expression plasmids were maintained by tryptophan selection. $2x10^4$, $2x10^3$, and $2x10^2$ cells were plated for each strain in duplicate and colonies were counted after 6 days of incubation at 30 °C. Percent survival was expressed as survival in the presence of canavanine compared to the same dilutions plated in the absence of canavanine.

**b. Chronic heat stress analysis**

Cell survival after chronic heat stress was determined as for canavanine survival, except that canavanine was omitted from the medium, and plates were incubated at 38.5°C for exactly 24 hours, followed by a shift to 22°C for 4 days to allow for colony development. The Ub derivative expression plasmids were maintained by tryptophan selection. As for the canavanine analysis, each strain was plated in duplicate for both the stress and unstressed experiments. Percent survival was expressed as the survival after 24 hours of heat stress as compared to the survival of the same dilution of each strain in the absence of heat stress.

**c. UV sensitivity**

UV survival was determined as for canavanine survival, except that canavanine was omitted from the plates, and cells were irradiated with 200 J per $m^2$ of UV (254 nm)

immediately following plating. Cells were then grown in the dark at 30°C for 4 days to allow for colony development. Percent survival is expressed as the number of colonies surviving after UV exposure compared to the number without UV exposure for each Ub derivative tested.

**d. Proteolysis of bulk canavanyl proteins *in vivo*.**

Protein turnover measurements were determined essentially as described by Hodgins *et al.* (1992) using the method of Seufert and Jentsch (1990) with slight modifications. Yeast cells (SUB60) carrying either the wild type Ub, or RRR.Ub expression plasmids, both with *TRP1* markers for selection, were used in this experiment. For a given yeast strain, a single fresh colony was inoculated into 30 ml of SD media supplemented with lysine (40 mg/liter), histidine (10 mg/liter), and uracil (2.4 mg/liter). The cultures were grown overnight at 30 °C while shaking. The mid log phase culture ($OD._{600}$ 0.2-0.8) was divided into two identical 20 ml cultures at 2 x $10^6$ cells per ml at 30°C. Ub and Ub.RRR gene expression was induced from the *CUP1* promoter by adding $CuSO_4$ to each culture (10 mM final concentration). These cultures were induced for 5 hours, after which time canavanine (final concentration 20 µg/ml) was added to one flask of each duplicated culture and grown for a further doubling period. Cells were then incubated with [$^{35}$S]methionine to label bulk cellular proteins. 75 µCi/ml of label was added to each culture and allowed to incubate for 5 minutes at room temperature with agitation. The cells were pelleted and the supernatant was carefully and fully removed by aspiration. All steps from this point on were carried out on ice. The radioactive pulse was chased with cold methionine (10 mM final concentration) for 5 minutes and washed four times to remove unincorporated label with the final pellet resuspended in 15 ml preheated (30°C) chase medium in flasks shaking in a controlled temperature (30°C) water bath. The first time point was taken at this time. Time points were taken at 0 min, 20, 40, 60, 90 and 120 minutes. At each time point, a 1 ml aliquot of cells was pelleted and 50 µl of the supernatant was taken from mid-tube far from the cell pellet and transferred into a scintillation vial. Total radioactivity was determined from 50 µl of culture taken directly from the 15 ml culture in the flask and added to a scintillation vial. Percent release was calculated as the cpm released [(supernatant)/(total radioactivity)]. Results were expressed as % radioactivity released into the medium from the yeast cells as a function of time

## 3.3 Results

### 3.3.1. The formation of K29 and K63 Ub-Ub linkages is dependent on specific ubiquitin conjugating enzymes.

In earlier studies, we had noticed that the coexpression of UbΔ (C-terminal deletion) and mUb (N-terminally epitope tagged Ub) in *UBI4* deleted cells dramatically increased the overall formation of Ub-protein conjugates formed *in vivo* as compared to cells overexpressing mUb alone (Figure 2.6, compare Lane C with remaining lanes). Since Ub is known to be conjugated to numerous cellular targets when cells are stressed (Raboy *et al.*, 1991; Parag *et al.*, 1987; Hilt *et al.*, 1993), these results suggested that UbΔ expression was itself a cellular stress. Under these conditions, in addition to high levels of multiUb conjugates, we also found an increase in the level of the Ub-Ub dimer conjugated though position 63. The existence of K63 linked Ub conjugates suggested that the E2 activity induced during this stress was capable of creating the K63 linkages. Consistent with the idea that UbΔ expression causes cellular stress, it has been shown that the expression of UbΔ in wild type cells can induce the expression of *UBI4* (J. Prendergast, unpublished data). Furthermore, since the *UBC4/5* genes are induced under conditions similar to those for *UBI4*, we reasoned that induced levels of UBC4 and UBC5 synthesis may be responsible for the increased catalysis of K63 linkages in these potentially stressed cells. Despite the limited knowledge of known intracellular degradative protein targets for UBC4 or UBC5, we believed that UBC4/5 were reasonable candidates for the formation of the K63 Ub-Ub linkage.

*A yeast strain deleted for the UBC4 and UBC5 genes cannot form the K29 Ub-Ub linkage, and is severely hindered in the ability to form the K63 linkage.*

Based upon the observation described above, the involvement of the UBC4/5 proteins in formation of K29, K48 and K63 Ub-Ub conjugates was determined *in vivo*. by determining if the absence of UBC4/5 proteins resulted in an associated loss of one or more Ub-Ub linkage. Both wild type (MHY501) and *UBC4/5* deleted cells (MHY508) were transformed with Ub derivatives blocked at the C-terminus by the Myc-epitope, (Ubm) for reasons outlined in detail in Chapter 2. These Ubm derivatives also had two lysines converted to arginine at any two of the lysines at positions 29, 48 and 63. The resulting Ub derivative proteins thus contained a single acceptable lysine for ubiquitination. Whole cell lysates from yeast cells transformed with the individual Ubm derivatives were then subject to Western analysis to determine which lysines can be used to form Ub-Ub conjugates in the absence of UBC4/5.

Indistinguishable from the *UBI4* deletion strain, (Figure 2.9), a Ub derivative which has arginines at positions 29, 48 and 63 (RRR.Ubm ) is conjugation defective and does not form a Ub-Ubm conjugate in the *ubc4/5Δ* strain (Figure 3.1a). Furthermore, in the *ubc4/5Δ* strain, the K29 conjugate is undetectable and the K63 conjugate is dramatically reduced (Figure 3.1b). Quantitation of the dimer abundance (relative to the monomer) shows that there is an approximately 16 fold drop in the amount of K63 conjugate observed in the mutant cells (1.4%) versus wild type cells (22%). By comparison, the K48 conjugate was virtually indistinguishable between wild type (15%) and mutant cells (14%). These results strongly suggest that UBC4 and UBC5 are involved in the formation of the K29 and K63 Ub-Ub linkages. The absence of the K29 linkage in the *ubc4/5Δ* strain suggests that this strain is insufficient for the formation of this linkage. In contrast to this, however, the detectable formation of the K63 Ub-Ub conjugate indicates that additional E2 enzymes remain in the *UBC4/5* deletion strain which can form this linkage.

*Overexpression of UBC4 in a ubc4/5Δ strain can return the K29 and K63 Ub-Ub conjugate to wild-type levels.*

To confirm that the UBC4/5 proteins are involved in the formation of the K29 and K63 Ub-Ub linkages, we restored UBC4 protein function by introducing a plasmid expressing the wild type UBC4 protein. This plasmid caused the levels of K29 and K63 Ub-Ub conjugates to return to those in wild type cells (Figure 3.2, compare to Figure 3.1). The ability of UBC4 protein to reconstitute the formation of the alternative Ub-Ub linkages in a *ubc4/5Δ* yeast strain suggests that UBC4 is sufficient, in the absence of UBC5, for the formation of these linkages. Furthermore, this observation implies that the loss of K29 and K63 conjugate formation is due to a dependence on UBC4 and is not the result of a peculiar mutation acquired by the strain.

*Yeast UBC1 and UBC2 (RAD6) deletion strains are defective for K63 Ub-Ub linkage formation*

To identify other Ub conjugating enzymes involved in the formation of the K63 Ub-Ub conjugate, a series of yeast strains deleted for specific E2 genes were tested for their respective abilities to form the K29, K48 and K63 dependent Ub-Ub linkages. This investigation was facilitated by the use of Ubm derivatives with single suitable lysine residues available for conjugation in an identical manner as was performed for the *ubc4/5Δ* Ub conjugate analysis.

Yeast strains deleted for *UBC1*, *UBC2* (*RAD6*), *UBC6*, and *UBC8* genes, as well as a temperature sensitive UBC3 (CDC34) yeast strain were investigated for their ability to

form the three distinct Ub-Ub linkages *in vivo*. Using the same methods described for the *ubc4/5Δ* strain, a noticeable effect on linkage formation was found for only two Ub conjugating enzymes. It was found that K63 dependent Ub-Ubm conjugates were present at a significantly lower abundance in yeast strains deleted for *RAD6* (*UBC2*) and *UBC1* genes as compared to the K63 Ub-Ubm conjugates formed by a wild type yeast strain (Figure 3.3) There was, however, only a slight change in the K29 or K48 Ub-Ubm conjugate level as compared to wild type. This data suggests that both RAD6 and UBC1 enzymes are able to form the K63 linked Ub-Ub conjugate in addition to the UBC4 protein and that the K29 dependent Ub-Ub conjugate is dependent on UBC4 activity.

*Overexpression of RAD6 and UBC1 in a ubc4/5Δ strain can augment the K63 linkage level but cannot form the K29 linkage.*

To further corroborate that the K63 linkages are being formed by these two Ub conjugating enzymes in addition to UBC4/5, we determined if RAD6 or UBC1 protein overexpression could restore the formation of K63 linkages when expressed in the *ubc4/5Δ* strain. It was found that the overexpression of RAD6 and UBC1 (Figure 3.4 b and c) could increase the level of K63 conjugates as compared to the *ubc4/5Δ* strain alone (Figure 3.4a). However, the K63 linkages did not apparently reach the levels previously observed for wild type yeast cells (Figure 3.1a).

### 3.3.2. Replacement of K63 in ubiquitin with a residue that cannot be ubiquitinated has a profound effect on stress survival.

The requirement of UBC4/5 for conjugating Ub to K29 and K63, coupled with the role of these enzymes in stress resistance, raised the question whether these linkages are necessary for stress resistance. We tested this idea by making use of the fact that, like the *UBC4/5* deletion strain, deletion of the polyUb gene, *UBI4*, results in extreme sensitivity to chronic heat or to the amino acid analog, canavanine (Finley *et al.*, 1987). If a given Ub-Ub linkage were important in this process, then the expression of a Ub derivative that could not make this linkage should not be able to suppress the *ubi4Δ* phenotype. Unlike all the Ub derivatives used in this and previous chapter, this series of expression vectors carried full length Ub cassettes with functional C-termini, allowing the expressed Ub proteins to function as either donors or acceptors in a multiUb chain (Appendix B). Each of the Ub derivatives used in the *in vivo* stress analysis was shown to be capable of being overexpressed (Figure 3.5) and the protein product was confirmed as Ub by immunoblotting whole cell lysate of yeast cells overexpressing the derivatives with the anti-Ub antibody (data not shown).

*Stress Sensitivity analysis.*

In this study, the ability and necessity of the three lysine residues shown to participate in Ub-Ub linkages to provide resistance when grown in the presence of amino acid analogs (Figure 3.6) or chronic heat (Figure 3.7) when expressed in the *ubi4Δ* yeast strain was tested. As shown, the expression of untagged Ub with replacements at either position 29 (R29 Ub) or 48 (R48 Ub) or both, was almost as effective as wild type Ub in restoring viability to *ubi4Δ* cells exposed to canavanine or chronic heat (Figure 3.6). R63 Ub, however, failed to restore viability to *ubi4Δ* cells with respect to either type of stress. This data suggests that the presence of K63 is necessary for resistance to canavanine in a *ubi4Δ* strain.

To determine if K63 was sufficient to provide stress resistance to canavanine or heat stress when neither the K29 nor K48 linkages could be formed, the stress resistance of the RRK Ub derivative was determined. As shown in Figure 3.6 and 3.7, the presence of the K63 position alone (RRK.Ub) was sufficient (in the presence of K6, K11, K27, and K33) to confer cell survival to near wild type levels. From these results, it appears that K63 is necessary for survival of *ubi4Δ* cells in the presence of the amino acid analog canavanine and chronic heat.

### 3.3.3. *ubi4Δ* cells expressing Ub derivatives that are linkage defective at any or all of positions 29, 48 or 63 do not exhibit phenotypes associated with other ubiquitin system deficiencies.

The previous results suggested a connection between the K63 Ub-Ub linkage and the stress functions of UBC4. The ability of RAD6, like UBC4, to form the K63 linkage suggested that the K63 linkage may also be involved in RAD6 function. One function of RAD6 is to provide UV resistance, a stress sensitivity that is easily monitored. Therefore, using a similar approach to the previous stress sensitivity analysis, we tested the effect on UV sensitivity of the *ubi4Δ* strain when expressing the various Ub derivatives.

As shown in Figure 3.8, there was no obvious increase in sensitivity to UV irradiation in the *ubi4Δ* strain regardless of the Ub derivative expressed. This suggests that in our system, the loss of the K63 dependent Ub-Ub linkage does not affect UV sensitivity. Therefore, despite the obvious phenotypic parallels observed for the R63 and *ubc4/5Δ* mutants, no such similar phenotype was observed for the R63 and *rad6Δ* mutants.

### 3.3.4. *ubi4Δ* cells overexpressing linkage defective ubiquitin derivatives do not exhibit a defect in protein degradation.

The attachment of K48 multiUb chains to proteins by Ub conjugating enzymes has been demonstrated to result in the efficient degradation of the targeted protein (Chau *et al.*, 1989; Gregori *et al.*, 1990). To investigate if the alternative Ub-Ub linkages also served as degradation signals, Ub derivatives were tested for their contribution to protein degradation *in vivo* on bulk and abnormal (canavanyl) proteins. We tested this by analyzing the effect of expressing a linkage defective Ub derivative (RRR.Ub) in cells deleted for the polyUb gene, *UBI4*. As shown in Figure 3.9, the rate of bulk protein turnover is unaffected by either the presence of canavanine, or the expression of a Ub derivative that is defective for linkage formation.

## 3.4 Discussion

This chapter describes the investigation of the role of the three Ub-Ub linkages *in vivo* in *S. cerevisiae*. Through previous experiments (Chapter 2), it was determined that the C-terminus of one Ub is able to be conjugated to other Ub moieties through three lysine positions; 29, 48 and 63. The identification of two novel Ub-Ub linkages formed *in vivo*, in addition to position 48 (Chapter 2), raised the question regarding the purpose of the alternative linkages *in vivo* as they are predicted to form structurally distinct conjugates which may act as discrete, intracellular signals. Our interest lay in determining if the alternative Ub-Ub linkage formed through lysine residues at position 29 and 63 had a similar role *in vivo* as the K48 Ub-Ub chain, or if their role was to form a different signal for use in alternative cellular functions.

Several Ub conjugating enzymes were identified which were required for isopeptide bond formation between Ub monomers at positions 29 or 63 *in vivo*. It was observed that the K29 Ub-Ub linkage appears to be entirely dependent on the Ub conjugating enzyme UBC4, although the overlapping complementation of UBC5 for the UBC4 protein does not allow us to directly assess the involvement of UBC5 itself. The formation of the K63 linkage, however, was found to depend on several Ub conjugating enzymes.

Yeast strains specifically deleted for *UBC1*, *UBC4/5* or *RAD6* exhibited significantly reduced levels of Ub-Ub conjugates joined through the K63 position (Figure 3.3), suggesting that these enzymes are capable of forming the K63 Ub-Ub linkage. No effect was observed for UBC8 or CDC34 deletion strains. Overexpression of UBC4 in the *ubc4/5Δ* strain completely reconstituted the formation of the K29 and K63 conjugates to wild type levels (Figure 3.2) indicating that the loss of the K29 and the decrease of K63 conjugates were a result of UBC4 absence. Conversely, overexpression of UBC1 or RAD6 in the *ubc4/5Δ* strain were unable to form the K29 conjugate but significantly

increased the formation of the K63 linkage (Figure 3.4). The simplest explanation for this data is that UBC4 is absolutely required for the K29 linkages, and significantly involved, with the additive contribution of UBC1 and RAD6, in the formation of the K63 linkage.

Our discovery of the involvement of the three E2 enzymes in K63 Ub-Ub linkage formation has recently been corroborated by another group (Finley *et al.*, 1994; Spence *et al.*, 1995). Their work has implicated UBC4, UBC1 and RAD6 in the formation of an unidentified Ub-protein conjugate formed *in vivo* (Spence *et al.*, 1995). Furthermore, this Ub-protein conjugate disappears when K63 Ub-Ub linkage formation is prevented by an arginine replacement (Spence *et al.*, 1995). These data, therefore, suggest that this unidentified Ub-protein conjugate is targeted by three E2 enzymes, UBC4, UBC1 and RAD6, in a K63 Ub-dependent manner. In agreement with our observations, it appears that K63 Ub linkages are formed by RAD6, UBC1 and UBC4. Likewise, a recent paper by Johnson *et al.* (1995) confirmed our results demonstrating that the formation of the K29 Ub-Ub conjugate *in vivo* is mediated by UBC4 and/or UBC5.

The requirement for the stress-inducible UBC4/5 protein for the formation of the two alternative Ub-Ub conjugates was significant in light of the previously determined ability of a Ub derivative mutated at position 48 (R48) to complement for the stress response (Finley *et al.*, 1994). The ability of R48 to complement for stress resistance suggested that the alternative Ub conjugates could be involved in this function of the Ub system, independently of the K48 Ub-Ub linkage. Upon investigating the K29 and K63 linkages for their contribution to stress resistance to heat and an amino acid analog, it was demonstrated that the K63 was necessary for stress resistance in the yeast strain tested (Figure 3.7).

Together, the heat stress and canavanine sensitivity experiments suggest that the ubiquitination of K63 plays a critical role in stress survival. Another interpretation of these results, however, is that the biological effects arising from K63 to R63 replacement results not from a failure to ubiquitinate this position as predicted, but from a subtle alteration of Ub structure that reduces its activity with respect to particular functions. Using this same line of reasoning, it would stand that the R29 Ub mutant would not possess this structural perturbation, explaining the ability of the R29 mutant to function as wild type Ub in the various stresses tested.

There are several arguments with respect to the present work that run counter to the structural perturbation viewpoint. First, the crystal structures of Ub (Vijay-Kumar *et al.*, 1987a) and the recently determined Ub dimer (Cook *et al.*, 1992) show that the K63 side chain makes no contact with the rest of the Ub molecule (Vijay-Kumar *et al.*, 1987b). Therefore, a simple lysine-to-arginine replacement at position 63 should have no effect on

overall structure. Secondly, although the arginine replacement at position 63 has no apparent effect on Ub structure, it is possible that K63 makes a critical contact with another protein that is affected upon its replacement. This latter possibility could manifest itself in either of two ways. First, expression of R63 Ub could produce a dominant-negative effect by titrating a protein that is necessary for stress resistance into an inactive R63Ub-protein complex. Such an event may cause a more severe stress sensitivity than the null phenotype of the *ubi4Δ* strain. The fact that the null mutant shows similar levels of stress sensitivity relative to the R63 Ub mutant (Figure 3.6 and 3.7) argues against this possibility.

In the second instance, K63 replacement may weaken an interaction with a protein that plays a critical role in stress resistance. Furthermore, such an interaction defect might be expected to show temperature sensitivity. This scenario might explain the effect of K63 substitution under conditions of chronic heat stress, specifically the extreme temperature sensitivity of the R63 Ub mutant. However, it is difficult to account for the exceptional canavanine sensitivity also exhibited by the mutant, even at temperatures as low as 22°C, which should not affect a temperature sensitive protein (data not shown). Based on our experiments alone, we cannot entirely rule out the possibility that the effect of R63 is not due simply to its failure to form K63 Ub conjugates, yet we feel that this explanation is unlikely.

The experimental observations made by Finley and Spence further support the argument that the effects observed *in vivo* for the R63 Ub mutant are related specifically to its failure to form K63 Ub-Ub linkages. Using a similar method to ours, these papers systematically looked at the effects of replacing each of the seven lysines in Ub with arginine, in a yeast strain which was deleted for all endogenous Ub genes, *UBI1-UBI4* (Finley *et al.*, 1994; Spence *et al.*, 1995). This strain, which is inviable without an acceptable source of Ub, was tested for survival after exposure to heat, canavanine, and UV, when expressing each of the Ub derivatives.

First, when present as the sole source of cellular Ub, R63 Ub and R29 Ub were able to fully support the growth of the *ubi1-4Δ* quadruple deletion strain. This confirms that in fact these mutants are functional proteins, because expression of a non functional Ub protein would not allow for cell viability (Finley *et al.*, 1994). Secondly, we had proposed that the inability of the R63 mutant to confer heat stress resistance in the *ubi4Δ* strain was possibly due to the Ub derivative being temperature sensitive itself rather than being specifically unable to provide heat stress resistance. Spence *et al.* recognized that the R63 Ub mutant was only slightly temperature sensitive in the *ubi1-ubi4Δ* strain (at an unreported temperature). A slight temperature sensitivity is not sufficient to explain the greater than five magnitude loss in cell viability upon R63 expression when exposed to heat

stress. Based on these observations, then, we suggest that the inability of the R63 Ub derivative to complement for the loss of the *UBI4* gene is a direct result of its inability to form the required K63 Ub-Ub linkage, and that the K63 Ub-Ub linkage is a critical linkage required in the yeast stress response.

A second function that the alternative Ub-Ub linkages were tested for was their role in the RAD6 DNA repair pathway, since we had determined that the K63 linkage was partially dependent on the expression of RAD6. We thus hypothesized that the R63 Ub mutants and the RAD6 deletion mutant may exhibit similar phenotypes, namely UV sensitivity, due to irreparable DNA damage. However, expression of Ub derivatives in the *ubi4Δ* strain failed to provide evidence to suggest that the individual linkages were involved in resistance to ultraviolet light, as the *ubi4Δ* strain showed no loss of viability after UV exposure, regardless of the Ub derivative expressed (Figure 3.8).

This is in contrast with results obtained by Spence and Finley, who observed that the overexpression of R63 Ub in a mutant yeast strain lacking any endogenous Ub resulted in cells that had a substantially increased UV sensitivity. Furthermore, this UV sensitivity was at a comparable level to that of a *rad6Δ* mutant (Spence *et al.*, 1995; Prakash *et al.*, 1989). The discrepancy between this work and our own data is presumably because of the presence of endogenous Ub in our yeast strain which was masking the phenotypes associated with the loss of specific linkages. Therefore, it appears as if the K63 linkages are also associated with the RAD6 DNA repair process.

The DNA repair pathway is a distinctly different process than the stress response pathway, yet the K63 linkage participates in both. This suggests that there exist at least two discrete cellular processes to which the K63 Ub-Ub linkage is critical, likely by targeting discrete proteins within each.

Lastly, the alternative linkages were tested for their involvement in protein degradation. We wanted to determine if the alternative Ub-Ub linkages formed through positions 29 and 63 were necessary for efficient protein turnover. Because UBC4 and UBC5 are known to be responsible for the ubiquitination and subsequent degradation of the majority of abnormal proteins in stressed cells (Seufert and Jentsch, 1990), we investigated the effect of bulk protein turnover in cells grown in the presence and absence of canavanine when the K29 and K63 Ub-Ub linkages were prevented. If the K63 and K29 linkages formed by UBC4/5 are required for the turnover of bulk protein, then the loss of these linkages should result in a decreased rate of canavanyl protein degradation.

We found that a Ub derivative incapable of forming any of the three Ub-Ub linkages showed no decrease in the rate of protein degradation as compared to wild type Ub when expressed in a *ubi4Δ* strain (Figure 3.9). One explanation for this result is that the

K29 and K63 Ub-Ub linkages do not serve as protein degradation signals in the cell. However, the presence of endogenous wild type Ub expressed from the remaining three Ub genes may have concealed a decrease in protein degradation. This possibility was thought to be particularly valid, as the RRR.Ub mutant used also lacked the K48 linkage. Because the K48 linkage has been shown to be responsible for targeting proteins for degradation through K48 multiUb chains (Johnson *et al.*, 1992; Chau *et al.*, 1989; van Nocker and Vierstra, 1993), the RRR.Ub mutant would be expected to exhibit a detectable loss in protein degradation through loss of the K48 multiUb chain.

Finley *et al.* (1994) found that the expression of R48Ub, in the same *ubi4Δ* strain used in our investigation, did result in a weak inhibition of protein turnover. This suggested that the endogenous Ub present in the *ubi4Δ* stain was not masking the degradative effects associated with a loss in degradation efficiency. It is not known why the RRR.Ub mutant did not decrease the protein turnover rate to at least the R48 level. One explanation may be attributed to experimental variability. Secondly, the loss of the two alternative Ub linkage positions in the RRR.Ub mutant may somehow counteract the R48 mutation. In this scenario, the targeting of K48 linkages results in protein degradation, and the targeting of K29 and/or K63 linkages results in protein stabilization. If this were true, then the RRR.Ub mutant would have lost the degradative K48 signal, and lost the K29 and/or K63 stabilization signal, resulting in a protein degradation rate indistinguishable from the wild type Ub protein turnover rate. Interestingly, it was recently demonstrated that an artificial linear Ub-protein target was stabilized in vivo when the K29 position was converted to arginine (Johnson *et al.*, 1995) suggesting that K29 linkages do contribute to protein degradation. It remains to be determined if this observation extends to natural targets that are not fused to a Ub moiety.

The expression of R63 Ub, as the sole source of Ub, in the *ubi1-4Δ* quadruple Ub mutant strain, however, did not affect the rate of protein degradation (Spence *et al.*, 1995). This implies that the K63-dependent Ub-Ub linkage is not necessary, or required, for bulk protein turnover. This does not conflict with our observation that a linkage defective Ub derivative mutated at position 29, 48 and 63 degrades proteins at a wild type rate. Yet, the explanation that the K29 linkage may act as an antagonist of protein degradation that balances the K48 multiUb chain degradation signal still exists. It has not been tested if K29 linked Ub conjugates function to stabilize proteins. In the *ubi1-ubi4Δ* strain, R29 Ub was not tested for protein degradation efficiency, but was able to fully complement for all Ub functions with only a slight decrease in cell proliferation rate (Spence *et al.*, 1995). This issue will remain unclear until natural targets of K29 chain assembly have been identified and tested for their stability.

In light of our data and that of Finley and Spence, clearly the model of how Ub relieves cellular stress should be revised. The role of Ub and Ub conjugation in overcoming heat and canavanine stress was thought to be to clear the cells of abnormal protein accumulation by targeting proteins for degradation (Burdon *et al.*, 1987; Hilt *et al.*, 1993; Parag *et al.*, 1987). The K48 multiUb chain has been established as a protein degradation signal (Johnson *et al.*, 1992; Chau *et al.*, 1989), as discussed above. Conversely, the K63 Ub-Ub linkage does not appear to be a signal in protein degradation, yet is necessary for yeast stress resistance. It may be that the majority of cellular proteins are degraded by K48 multiUb chains created by UBC4/5, but the targeting of K63 Ub-Ub linkages to discrete cellular proteins results in stress resistance.

In summary, the different linkages formed between Ub monomers appear to behave as independent signals, with the K48 Ub-Ub linkage having a role in signaling proteins for degradation and the K63 Ub-Ub linkage having a role in the yeast stress response. These investigations did not reveal a function for the K29 Ub-Ub linkage, yet a recent paper has provided evidence to suggest that K29 can function as a degradation signal (Johnson *et al.*, 1995).

A greater understanding of the mechanisms used by the E2 class of enzymes in the Ub-dependent proteolytic pathway requires that additional cellular proteins associated with, and affected by, the E2 be identified. In this manner, we may more fully understand the mechanism of target selection, the timing of protein targeting, and the choice of Ub-Ub linkages used. As K48 multiUb chains have been shown to be involved in protein degradation by its recognition by several subunits of the 26S proteasome, it may also be that K63 Ub-Ub conjugates are recognized by different cellular factors to carry out its apparent roles in DNA repair and stress resistance. In the next chapters, we have set out to create conditional mutants of a Ub conjugation enzyme to allow us to identify directly interacting proteins through a genetic screen.

FIGURE 3.1

**A yeast *UBC4/5* deletion strain is deficient in K29 and K63 dependent Ub-Ub conjugate formation.**

Shown is an SDS anti-Myc Western blot of total protein from yeast cells coexpressing C-terminal tagged Ub acceptor (Ubm) with untagged wild type Ub. These derivatives are expressed in wild type cells (Part a) or in cells deleted for the *UBC4* and *UBC5* genes (*ubc4/5Δ*, Part b). For Ubm derivatives, only lysines identified to be targets of ubiquitination (circled positions) have been mutated to Arginine in the combinations shown (boxed positions). Numbers mark the position of lysines (K) present in the wild type Ub sequence (Ubm.K+). The Myc epitope is shown as a black box. For gels, the position of monomeric, dimeric, and trimeric Ubm and Ubm conjugates are as indicated. Also indicated are the positions of molecular weight marker standards. *Gel lanes:* +, O, 29, 48 and 63 refer to Ubm derivatives K+, RRR, KRR, RKR and RRK respectively.

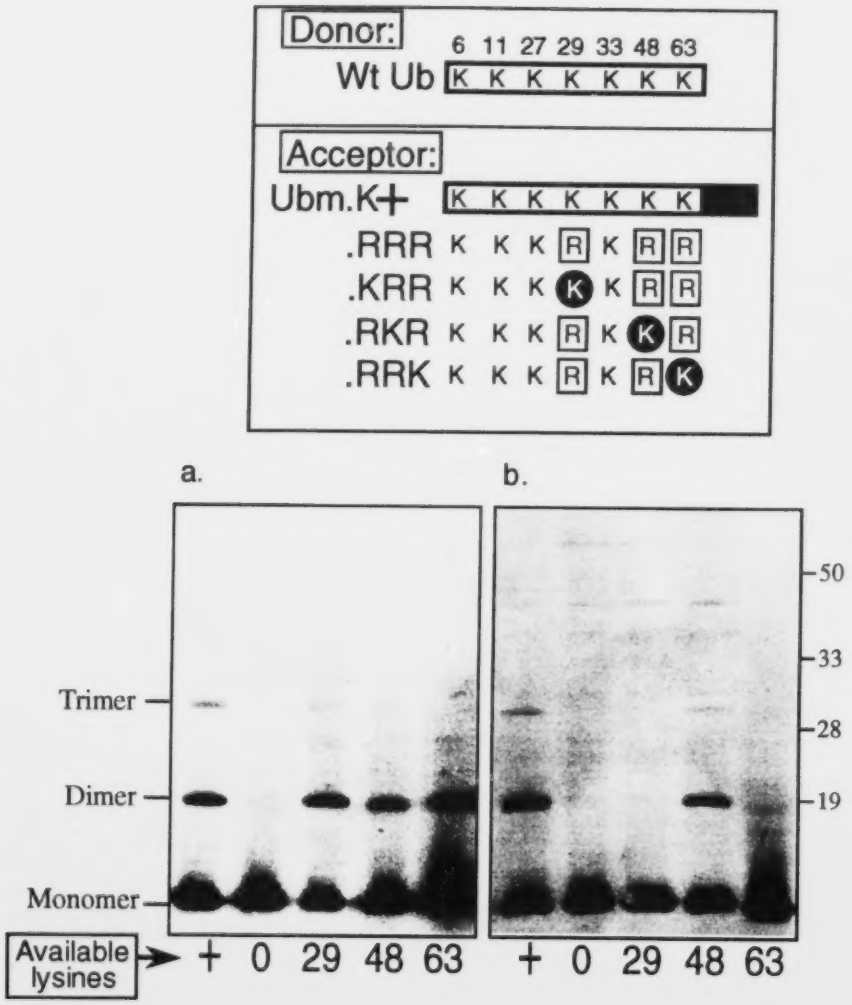

Donor:
6 11 27 29 33 48 63
Wt Ub K K K K K K K
Acceptor:
Ubm.K+ K K K K K K K
.RRR K K K R K R R
.KRR K K K K K R R
.RKR K K K R K K R
.RRK K K K R K R K
a.
b.
Trimer
Dimer
Monomer
50
33
28
19
Available lysines
+ 0 29 48 63
+ 0 29 48 63

FIGURE 3.2

**K29 and K63 Ub-Ub conjugates are dependent on *UBC4* expression**

Shown is an SDS anti-Myc Western blot of total protein from yeast cells coexpressing C-terminal tagged Ub acceptors (Ubm) in combination with untagged wild type Ub (Part a) or a UBC4 expression plasmid (pUBC4, Part b). For Ubm derivatives, only lysines identified to be targets of ubiquitination (circled positions) have been mutated to arginine in the combinations shown (boxed positions). Ubm derivatives are expressed in a strain wild type (Part a) or deleted for the *UBC4* and *UBC5* genes (*ubc4/5Δ*, Part b). Numbers mark the position of lysines (K) present in the wild type Ub sequence (Ubm.K+). The Myc epitope is shown as a black box. For gels, the position of monomeric and dimeric Ubm and Ubm conjugates are as indicated. Part a is a duplicate of Figure 3.1a, and is included as a reference. Also indicated are the positions of molecular weight marker standards. *Gel lanes:* +, O, 29, 48 and 63 refer to Ubm derivatives K+, RRR, KRR, RKR and RRK respectively.

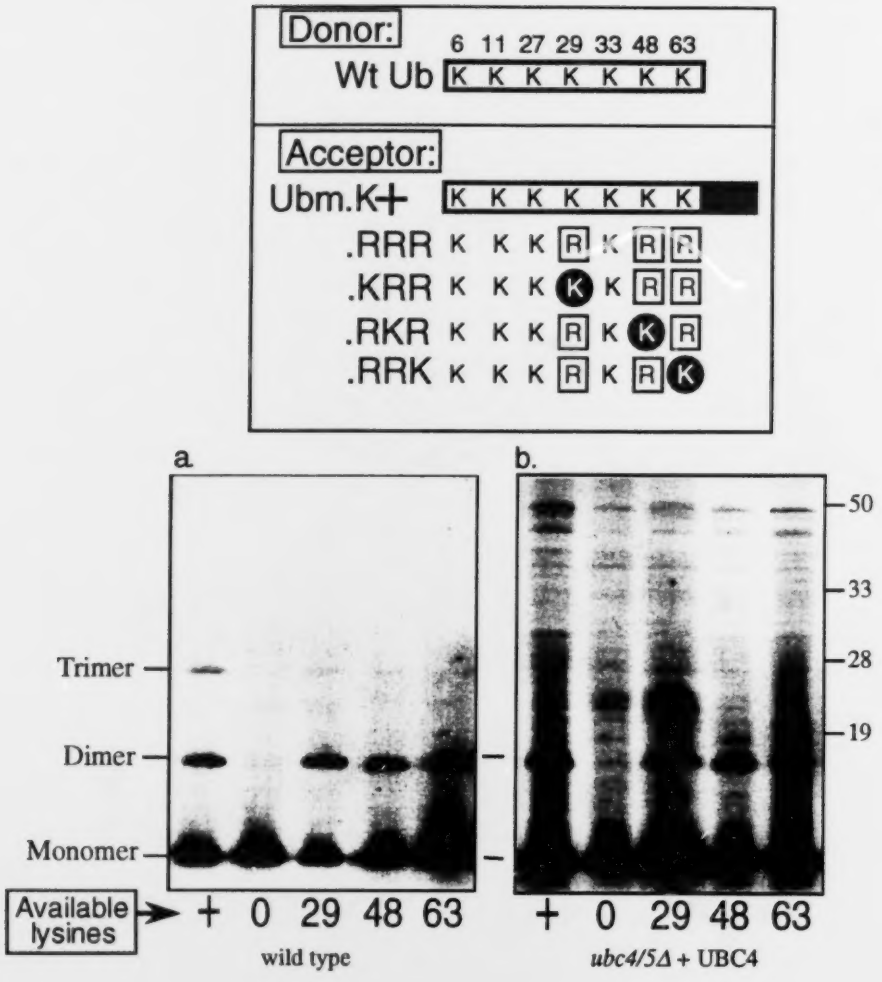

Donor:
6 11 27 29 33 48 63
Wt Ub K K K K K K K
Acceptor:
Ubm.K+ K K K K K K K
.RRR K K K R K R R
.KRR K K K K K R R
.RKR K K K R K K R
.RRK K K K R K R K
a.
b.
Trimer
Dimer
Monomer
50
33
28
19
Available lysines
+ 0 29 48 63
+ 0 29 48 63
wild type
ubc4/5Δ + UBC4

FIGURE 3.3

**K63 Ub-Ub conjugate levels are decreased in yeast strains deleted for either the *UBC1* or *RAD6* genes.**

Shown is an SDS anti-Myc Western blot of total protein from yeast cells coexpressing C-terminal tagged Ub acceptors (Ubm) with untagged wild type Ub. For Ubm derivatives, only lysines identified to be targets of ubiquitination (circled positions) have been mutated to Arginine in the combinations shown (boxed positions). Ubm derivatives are expressed cells that are wild type (Part a), or deleted for the *UBC1* gene (*ubc1Δ*, Part b), or deleted for the *RAD6* gene (*RAD6Δ*, Part c). Numbers mark the position of lysines (K) present in the wild type Ub sequence (Ubm.K+). The Myc epitope is shown as a black box. For gels, the position of monomeric and dimeric Ubm and Ubm conjugates are as indicated. Part a is a duplicate of Figure 3.1 a, and is included as a refence. Also indicated are the positions of molecular weight marker standards. *Gel lanes:* +, O, 29, 48 and 63 refer to Ubm derivatives K+, RRR, KRR, RKR and RRK respectively.

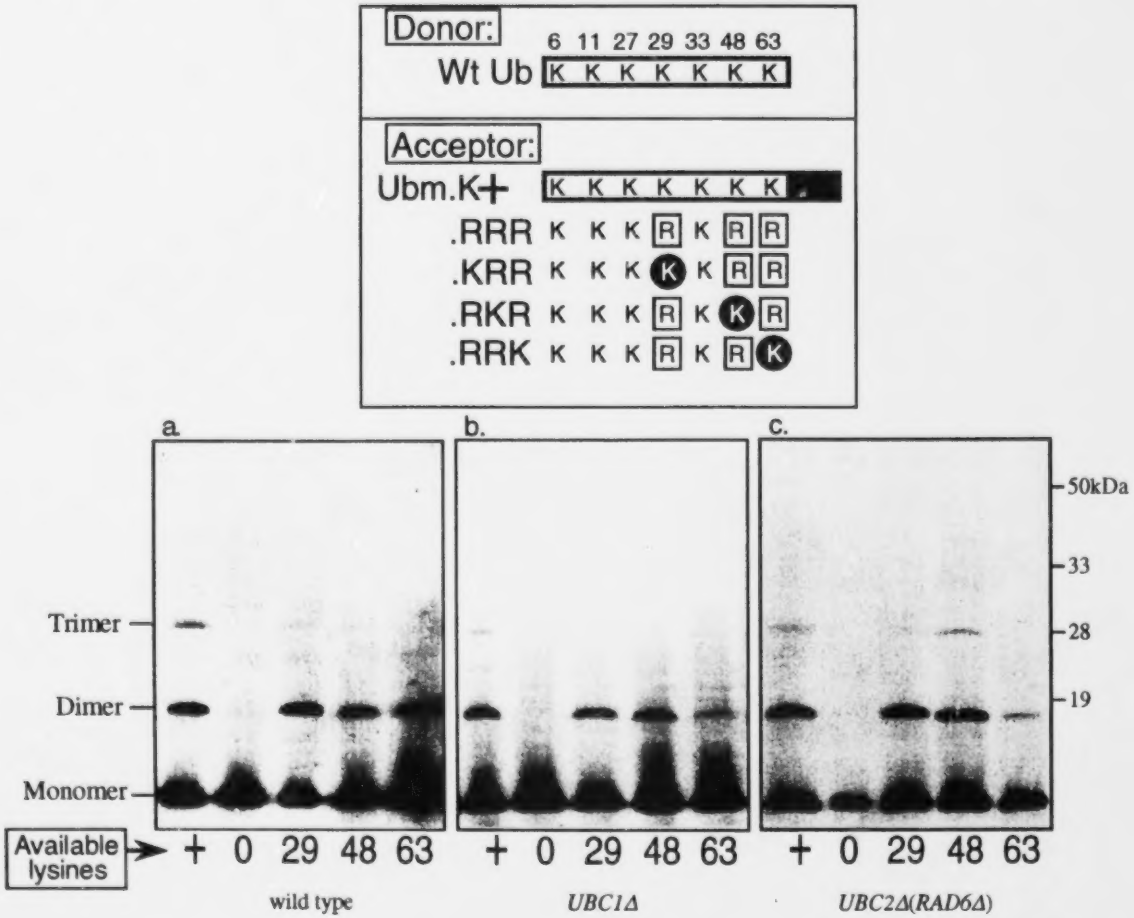
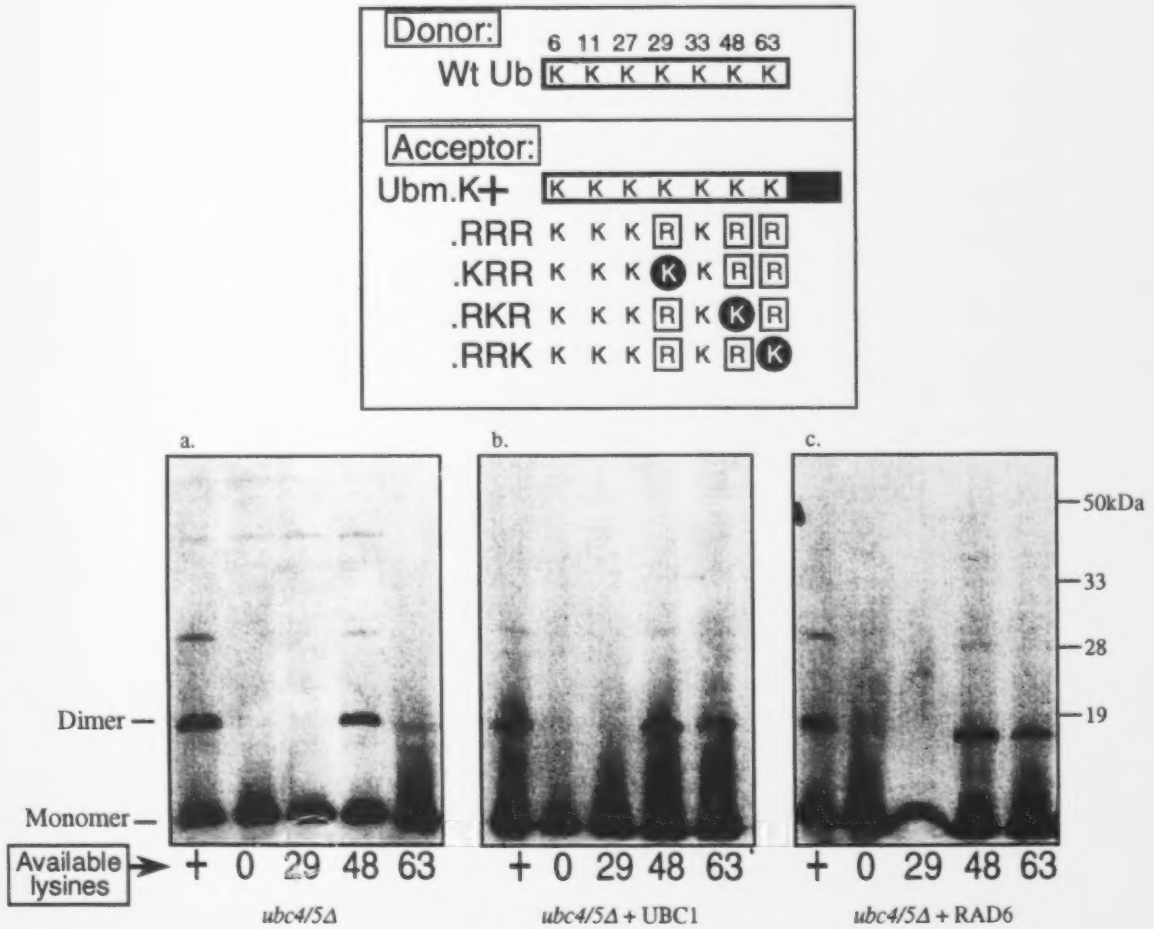

Donor:
6 11 27 29 33 48 63
Wt Ub K K K K K K K
Acceptor:
Ubm.K+ K K K K K K K
.RRR K K K R K R R
.KRR K K K K K R R
.RKR K K K R K K R
.RRK K K K R K R K
a.
b.
c.
50kDa
33
28
19
Trimer
Dimer
Monomer
Available lysines
+ 0 29 48 63
+ 0 29 48 63
+ 0 29 48 63
wild type
UBC1Δ
UBC2Δ(RAD6Δ)

FIGURE 3.4

**K63 Ub-Ub conjugate levels in the *ubc4/5Δ* strain are increased by overexpression of *UBC1* or *RAD6*.**

Shown is an SDS anti-Myc Western blot of total protein from yeast cells coexpressing C-terminal tagged Ub acceptors (Ubm). For Ubm derivatives, only lysine identified to be targets of ubiquitination (circled positions) have been mutated to arginines in the combinations shown (boxed positions). Ubm derivatives are expressed in cells that are wild type and coexpress untagged wild type Ub (Part a) or deleted for the *UBC4* and *UBC5* genes (*ubc4/5Δ*) in combination with *UBC1* (Part b) or *RAD6* (Part c) expression plasmids. Numbers mark the position of lysines (K) present in the wild type Ub sequence (Ubm.K+). The Myc epitope is shown as a black box. For gels, the position of monomeric, dimeric, and trimeric Ubm and Ubm conjugates are as indicated. Part a is a duplicate of Figure 3.1 a, and is included as a refence. Also indicated are the positions of molecular weight marker standards. *Gel lanes:* +, O, 29, 48 and 63 refer to Ubm derivatives K+, RRR, KRR, RKR and RRK, respectively.


Donor:
6 11 27 29 33 48 63
Wt Ub K K K K K K K
Acceptor:
Ubm.K+ K K K K K K K
.RRR K K K R K R R
.KRR K K K K K R R
.RKR K K K R K K R
.RRK K K K R K R K
a.
b.
c.
50kDa
33
28
19
Dimer
Monomer
Available lysines
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+ 0 29 48 63
+ 0 29 48 63
ubc4/5Δ
ubc4/5Δ + UBC1
ubc4/5Δ + RAD6

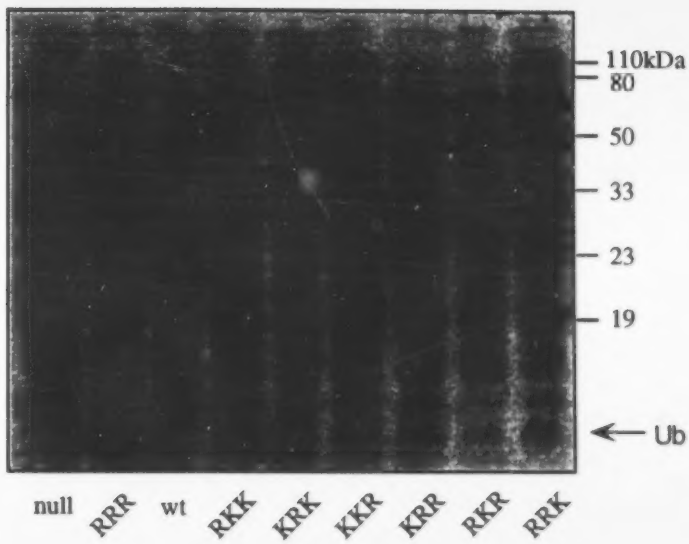



FIGURE 3.5

**The full length Ub derivatives are overexpressed *in vivo* in a *UBI4* deletion strain.**

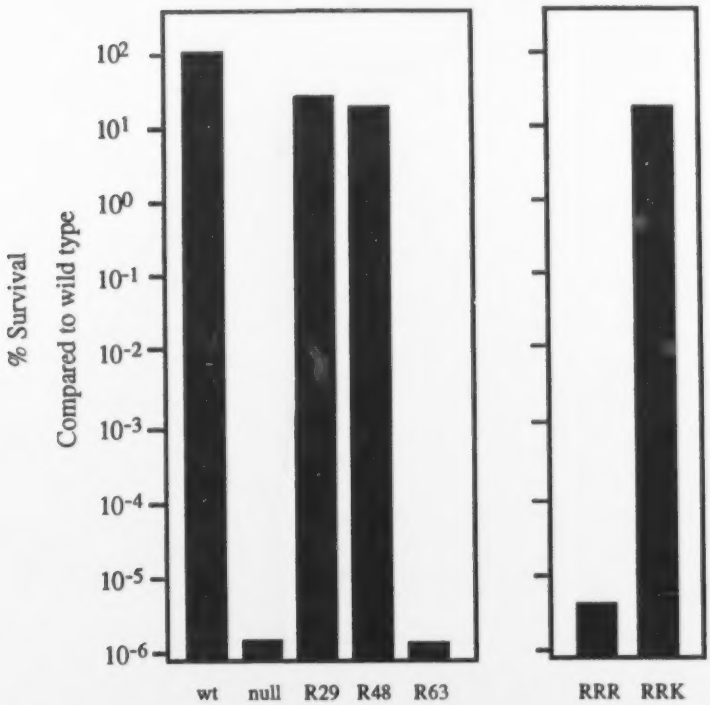
Shown is a Coomassie stained 18% acrylamide gel of whole cell lysates from the *UBI4* deletion strain overproducing various Ub derivatives. The strains harvested include the *ubi4Δ* strain alone (SUB60, null), or expressing wild type (wt) Ub and seven full length Ub derivatives with various lysine-to-arginine substitutions. The Ub derivatives used were altered only at the lysines identified to be targets of ubiquitination (positions 29, 48 and 63). As depicted in this Figure, the first, second, and third letters in the three letter nomenclature of the seven Ub derivatives refers to position 29, 48 and 63 of Ub, respectively. The position of the Ub protein is indicated by an arrow.

FIGURE 3.6

**An R63 Ub mutant is unable to complement for canavanine sensitivity in a *UBI4* deletion strain.**

Survival of the *UBI4* deleted yeast strain expressing different untagged Ub derivatives is represented as a percentage of survivors relative to cells expressing wild-type Ub (wt) as determined in plating assays under the canavanine conditions described in Materials and Methods. R29, R48 and R63 refer to Ub derivatives with single lysine-to-arginine mutations at the positions indicated. RRR is a Ub derivative with arginine replacements at positions 29, 48 and 63 and RRK has relacements at positions 29 and 48. Null refers to cells containing a non-Ub plasmid and serves as a negative control. Also shown are the survival percentages of each strain under stressed conditions as compared to unstressed conditions.

Canavanine Stress




% Survival of unstressed control.

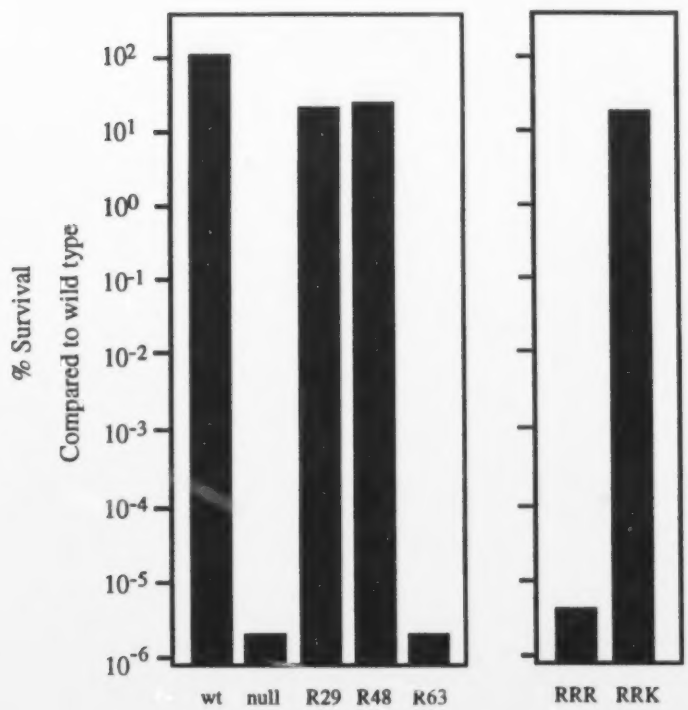
| Plasmid | wt | null | R29 | R48 | R63 | RRR | RRK |
|---|---|---|---|---|---|---|---|
| % | 75 | $0.8^{-6}$ | 47.9 | 44.5 | $0.8^{-6}$ | $6.25^{-6}$ | 70.5 |

FIGURE 3.7

**An R63 Ub mutant is unable to complement for heat stress in a *UBI4* deletion strain.**

Survival of the *UBI4* deleted yeast strain expressing different untagged Ub derivatives is represented as a percentage of survivors relative to cells expressing wild-type Ub (wt) as determined in plating assays under the heat stress conditions described in Materials and Methods. R29, R48 and R63 refer to Ub derivatives with single lysine-to-arginine mutations at the positions indicated. RRR is a Ub derivative with arginine replacements at positions 29, 48 and 63 and RRK has relacements at positions 29 and 48. Null refers to cells containing a non-Ub plasmid and serves as a negative control. Also shown are the survival percentages of each strain under stressed conditions as compared to unstressed conditions.

Heat Stress




% Survival of unstressed control.

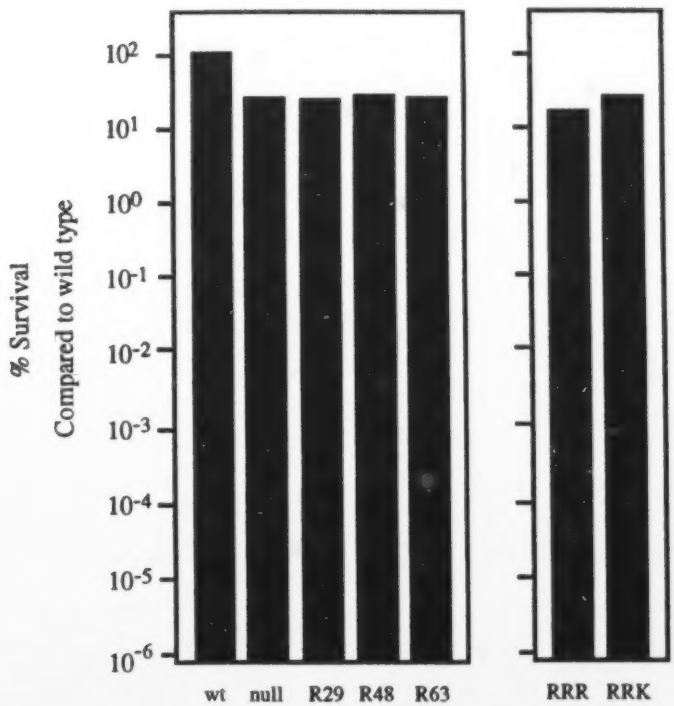
| Plasmid | wt | null | R29 | R48 | R63 | RRR | RRK |
|---|---|---|---|---|---|---|---|
| % | 79 | $1.59^{-6}$ | 34.7 | 36.9 | $1.59^{-6}$ | $6.25^{-6}$ | 38.2 |

FIGURE 3.8

**The *UBI4* deletion strain expressing various Ub mutants is not sensitive to UV irradiation.**

Survival of the *UBI4* deleted yeast strain expressing different untagged Ub derivatives is represented as a percentage of survivors relative to cells expressing wild-type Ub (wt) as determined in plating assays under the UV conditions described in Materials and Methods. R29, R48 and R63 refer to Ub derivatives with single lysine-to-arginine mutations at the positions indicated. RRR is a Ub derivative with arginine replacements at positions 29, 48 and 63 and RRK has relacements at positions 29 and 48. Null refers to cells containing a non-Ub plasmid and serves as a negative control. Also shown are the survival percentages of each strain under stressed conditions as compared to unstressed conditions.

UV Sensitivity




% Survival of unstressed control.

| Plasmid | wt | null | R29 | R48 | R63 | RRR | RRK |
|---|---|---|---|---|---|---|---|
| % | 80 | 60 | 60 | 62 | 62 | 49 | 75 |

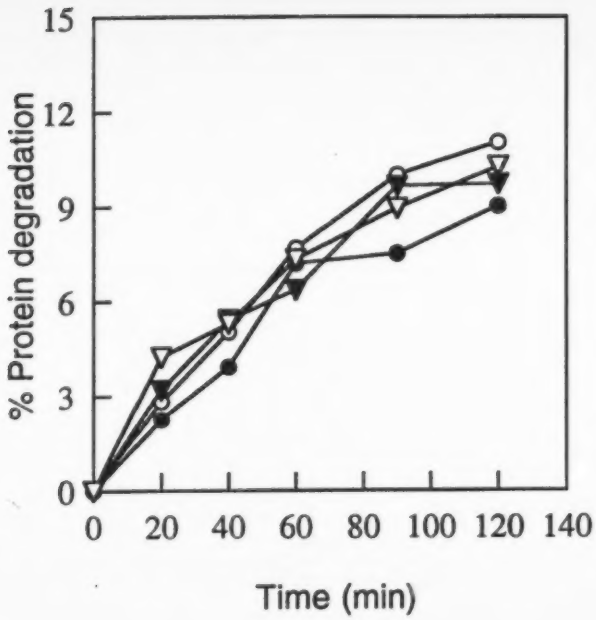



FIGURE 3.9

**A linkage defective Ub mutant does not affect the rate of canavanyl protein degradation.**

Protein turnover in yeast cells expressing wild type Ub (circles) or RRR.Ub (triangles) was measured in the presence (open symbols) or absence (closed symbols) of canavanine as described in Materials and Methods. Protein degradation is expressed as a percentage of the total incorporated radioactivity released from cells as a function of time.

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# CHAPTER 4. A site-directed approach for the construction of stress sensitive and growth defective derivatives of UBC4.

## 4.1 Introduction

In the previous Chapter, several lines of evidence were presented that correlated the UBC4/5 dependent formation of lysine (K) 63 Ub-Ub linkages with the stress response. It is generally believed that UBC4 and UBC5 function in the elimination of damaged or abnormal proteins that arise through normal metabolic activity or from exposure to environmental stress. It is also believed that the sensitivity of *ubc4/5Δ* strains to chronic heat stress and canavanine, and their slow growth reflect a failure of these cells to degrade damaged or abnormal proteins via Ub dependent proteolysis (for review see Seufert and Jentsch, 1991; Jentsch, 1992).

Despite the reasonableness of this belief, there is little direct evidence to distinguish between the possibility that these three different phenotypes are actually attributable to a common biochemical pathway versus a simple reflection of distinct UBC4/5 functions. Such a question could be potentially resolved by examining the phenotypic consequences of different mutations within the coding sequence of the UBC4 protein. Mutations that strongly affected one phenotype but not another would provide conclusive evidence that different phenotypes reflected defects in distinct UBC4-related biochemical pathways.

Furthermore, mutations that displayed specificity towards a subset of phenotypes would provide effective tools for the isolation of gene products that facilitated UBC4 function within a given pathway. It has long been recognized that the overexpression of proteins that physically interact with a temperature sensitive (*ts*) polypeptide can reverse its thermal lability through associations (see for example Prendergast *et al.*, 1995). Proteins that interact with UBC4 could, therefore, be selected using a high copy suppression strategy in which a high copy yeast library was introduced into yeast followed by selection for plasmids that reverted the temperature sensitivity of a given UBC4 mutation.

With these ideas in mind, we created and availed ourselves of several UBC4 *ts* mutations whose phenotypic characterization is described here. We selected mutations that substituted single amino acids on the surface of UBC4 as judged from its recently determined crystal structure (Cook *et al.*, 1993), thereby increasing the chances of selectively disrupting key protein-protein interactions associated with one UBC4 function or another. In addition we restricted these analyses to conserved amino acid positions whose substitutions in other E2s resulted in effective *ts* phenotypes (Ellison *et al.*, 1991;

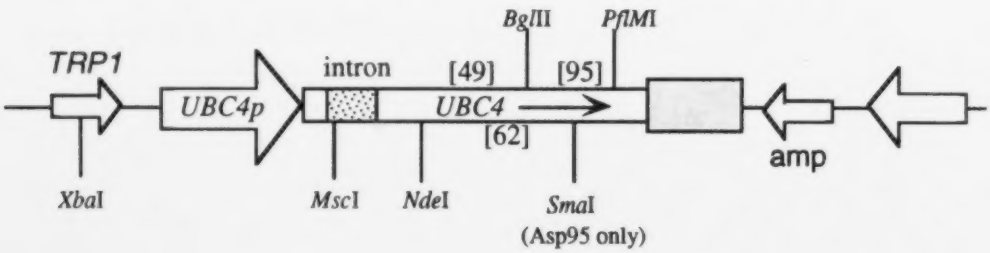
Seufert *et al.*, 1995; M. Goebl, unpublished results). Based on these restrictions, we constructed three UBC4 mutants corresponding to single amino acid substitutions at positions 49, 62 and 95 (Figure 4.1). We also tested a RAD6 derivative containing two amino acid substitutions (Asp65 to Phe, Tyr82 to Asn) that conferred partial UBC4 function to the *ubc4/5Δ* strain without concomitant loss of its RAD6 function, referred to as the RAD6/UBC4 chimaera because of its dual function (Chantelle Gwozd, unpublished results). The results of these studies indicate that the observable UBC4 phenotypes are not different manifestations of the same biochemical pathway. They also identified a mutant that proved to be suitable for the genetic suppression screen described above.

## 4.2 Materials and Methods

### Yeast expression Vectors

a. Low copy yeast plasmids; CEN/ARS vectors.

The parental yeast vectors used in the growth and stress experiments had the ampicillin marker for plasmid maintenance in *E. coli* ( a yeast shuttle vector), and either the *URA3* or *TRP1* marker for selection in *S. cerevisiae*. The low copy parental vector used in the construction of *UBC4* point mutants is shown below. A partial linear map indicating relevant regions of the vector and key restriction sites used in the construction of the *UBC4* mutants accompanies the description of the vector. The map is not to scale. Abbreviations are as follows: *UBC4p*, the genomic *UBC4* promoter; *TRP1*, 5' P-ribosyl-anthranilate isomerase gene; CEN/ARS, centromere-autonomous replication sequences causing the plasmid to exist at one or two copies per cell; intron, the 95 base pair (bp) intron present in the *UBC4* gene, whose position is schematically shown by the stippled box. The relative position of the three point mutations (49, 62 and 95) are indicated on the diagram with respect to relevant restriction enzyme sites.



**General method for the construction of the UBC4 point mutants.**

The three UBC4 protein derivatives were made by introducing single codon changes into a wild type *UBC4* gene present on an expression plasmid. Mutations were introduced by PCR and required two stages, the first to generate a portion of the *UBC4* gene carrying the mutation, and a second round of PCR to generate the full length *UBC4* mutants. The final PCR products were introduced onto a low copy CEN/ARS *TRP1* yeast vector for expression from the endogenous *UBC4* promoter *in vivo*. The internal sequences of a wild type *UBC4* gene already present on a CEN/ARS vector were replaced by the mutated *UBC4* sequences. Specifically, the large *Pfl*MI-*Msc*I fragment of the wild type low copy number plasmid was ligated with the *Pfl*MI-*Msc*I digested PCR products. All three *UBC4* gene mutants carry the intron sequences.

**a. Construction of arginine (Arg) 49 UBC4.**

The glycine 49 codon (GGT) was converted to an arginine codon (CGT) by PCR. The PCR template was the wild type *UBC4* sequence which included the intron sequences. The 5' PCR oligonucleotide Ter20;
(5'- CCTGCCGATTCCCCATATGCCGGGCGTCTTTTC-3') introduced the mutations, and retained an *Nde*I restriction site present in the wild type UBC4 sequence (underlined). The two base pairs changed from wild type are denoted as bold face nucleotides. The altered G destroys an *Nae*I restriction site normally present in wild type *UBC4* sequences, and was used as a selectable alteration in the PCR product for successful mutation. The 3' primer used in the PCR reaction was MEL29;
(GCTAGGTACCTCATACAGCGTATTTCTTTGTCC) which is complementary to the extreme 3' sequences of the *UBC4* gene. This oligonucleotide does not introduce any sequence alterations, but does add a *Kpn*I restriction site after the STOP codon.

The resulting PCR product did not have the 5' *UBC4* coding sequences, so these were introduced by the ligation of wild type *UBC4* 5' sequences to the 3' sequences of the *UBC4* mutant. Wt *UBC4* on an expression plasmid was digested with *Nde*I-*Bgl*II, and the dephosphorylated large fragment was ligated with *Nde*I digested PCR product. Full length Arg49 *UBC4* sequences were obtained by PCR using MEL28;
(AAACATGAGCTCTTCTAAACGTATTGC), which is complementary to the extreme 5' end of the *UBC4* gene, and MEL29, described above. Neither primer introduces sequence alterations in the *UBC4* gene, but the MEL28 primer does introduce a *Sst*I restriction site 5' at codons 2 and 3 of the *UBC4* coding sequence. This full length PCR product was then introduced onto a low copy *TRP1* based yeast plasmid as described above. This vector was confirmed as correct by double stranded DNA sequencing.

**b. Construction of serine (Ser)62 UBC4.**

The proline 62 codon (CCA) was converted to a serine codon (TCA) by PCR. The PCR template was the wild type *UBC4* sequence which includes the intron sequences. The 3' PCR oligonucleotide Ter21;
(5'-GTGAAGGAGATCTTTGGTGG[TTTAAA]TGAGTAGTCGG) introduced the sequence mutations, and retained the *Bgl*II restriction site present in the wild type *UBC4* sequence (underlined). The three base pair changes from wild type are denoted as bold face nucleotides. The first 5' altered T created the Serine 62 codon. The two additional mutations no not change amino acid codons, but do introduce restriction sites resulting in the creation of a new *Dra*I restriction site (marked by brackets). The presence of the new *Dra*I site allowed us to distinguish between the *UBC4* mutants and wild type sequences. The 5' primer used in the PCR reaction was MEL28 (described above).

*Generation of full length Ser62 UBC4.*

The resulting PCR product did not have the 3' *UBC4* sequences, so these were introduced by the ligation of wild type *UBC4* 3' sequences to the 5' coding sequences of the *UBC4* mutant. Wild type *UBC4* on an expression plasmid was digested with *Xba*I-*Bgl*II, and the dephosphorylated large fragment was ligated with *Bgl*II digested PCR product. Full length Ser62 *UBC4* sequences were obtained by PCR using MEL28 and MEL29, described above. Neither primer introduced sequence alterations in the *UBC4* gene. This full length PCR product was then introduced onto a low copy *TRP1* based yeast plasmid as described above. This vector was confirmed by double stranded DNA sequencing.

**c. Construction of Aspartate (Asp)95 UBC4.**

The serine 95 codon (TCT) was converted to an aspartate codon (GAT) by PCR. The PCR template was a wild type *UBC4* sequence lacking the intron sequences (which had been specifically deleted using PCR), but which had a unique *Sma*I site introduced at codons 96 and 97. The proline codon 96 (CCA) was silently altered to (CCC), however the alanine codon 97 (GCT) was conservatively replaced with a glycine codon (GGG). The necessity of having the *Sma*I site present in this *UBC4* sequence was to provide a unique restriction site adjacent to the Asp95 mutation. The *UBC4* template carrying the glycine 97 codon expressed a protein which behaved indistinguishably from that of wild type UBC4 (data not shown). To introduce the aspartate sequence mutations at position 95, a 3' PCR oligonucleotide Ter22 was used,

(5'-CCTTCGATCGAGTTAG[CCCGGG]ATCCCATTGATCC). The two base pair changes from wild type are denoted as bold face nucleotides and create the Asp (TCT) codon as well as introducing a unique *BamH*I restriction site (underlined), useful in distinguishing between mutated and wild type *UBC4* sequences. The *Sma*I restriction site present in the primer is bracketed. The 5' primer used in the PCR reaction was MEL28 (described above).

The resulting PCR product did not have the 3' *UBC4* coding sequences, so these were introduced from the *UBC4* vector described above which lacks the intron as well as having a unique *Sma*I site introduced at the expense of a Gly-to-Ala conversion. This vector was digested with *Sma*I-*Bgl*II, and the dephosphorylated large fragment was ligated with *Sma*I digested PCR product. The full length *UBC4* derivative was selected by PCR using MEL28 and MEL29, described above.

This PCR product could not be directly introduced by a *Msc*I-*PflM*I digest onto a low copy CEN/ARS vector because the intron sequences, and the accompanying *Msc*I site, were absent. Therefore, the intron sequences were reintroduced at their normal 5' location within the *UBC4* gene. A wild type *UBC4* gene (with the intron sequences) present on a low copy vector was digested with *Bgl*II-*PflM*I and the small fragment encoding for the 3' portion of the *UBC4* gene was removed. The *Bgl*II-*PflM*I digested Asp95 UBC4 PCR product was used to replace this fragment, resulting in a *UBC4* gene with the 5' intron, the unique *Sma*I restriction site, and the 3' Asp95 mutation. This vector was confirmed as correct by double stranded sequencing of the entire gene.

**d. Construction of a RAD6/UBC4 chimaera.**

The first step in the construction of the RAD6/UBC4 chimaera was to create a *RAD6Δ* cassette (constructed by Todd Gwozd). The details of the construction are as follows. A *RAD6* gene, deleted for the acidic C-terminus (denoted as *RAD6Δ*) was altered so that the N-C-, and internal sequences were divided roughly into three subdomains by the introduction of two unique restriction sites for purposes not relevant here. The only amino acid changes introduced by these sequence alterations are as follows: threonine 99 was converted to a glycine to introduce a *Sma*I site, threonine 59 was converted to an alanine to introduce a *Kas*I site. Mutations were introduced by PCR, and the product was able to fully function in a *RAD6Δ* yeast strain. The gene cassette was also confirmed by double stranded sequencing.

The RAD6/UBC4 chimaera was constructed by Chantelle Gwozd. The purpose of making this was to define the residues specifically involved in UBC4 function by transferring UBC4 activity to RAD6. Residues in RAD6 were changed to those specific

for UBC4. The RAD6/UBC4 chimaera used in this Chapter contains two amino acid conversions in the *RAD6Δ* cassette described above. Specifically, the phenylalanine (Phe) codon (position 63) and the asparagine (Asn) codon (position 80) in *UBC4* were introduced at the corresponding sites in the *RAD6Δ* gene, resulting in a RAD6Δ Phe65 Asn82 protein. These mutations were introduced by PCR in two separate PCR reactions whose products were then ligated together. The 5' fragment of the *RAD6Δ* gene had the Phe65 mutation introduced by PCR using the 3' primer MEL 114;
(AGTGAAATGTTTCATCCCAATGTTAACGCAAATGG) and MEL1 (specific for *RAD6* sequences) as the 5' primer. The 3' fragment of the *RAD6* gene had the Asn82 mutation introduced by PCR using the 5' primer MEL115;
(GATGAAACATTTCACTCAAAAAATTTGACATGCGGTGGTTTAAAAGGGATA)
with the 3' primer MEL23 (complementary to the 3' portion of the RAD6 gene). MEL114 and MEL115 introduced *Xmn*I restriction sites at the 3' and 5' ends of their respective PCR products. Digestion with *Xmn*I followed by ligation allowed for selection of the entire mutated *RAD6Δ* gene by PCR using MEL1 and MEL23. These primers included both the *Kpn*I and *Sst*I restriction sites at the extreme 3' and 5' ends of the *RAD6Δ* gene cassette. After digestion with these two enzymes, the mutated cassette was placed into a *TRP1* high copy yeast vector under the control of the *CUP1* promoter in a manner identical to previous E2 gene transfer to *TRP1* expression plasmids (See Materials and Methods, Chapter 3). This gene sequence was confirmed by double stranded DNA sequencing.

**_UBC4_ and _RAD6Δ_ derivative gene expression from high copy number plasmids.**

The linkage analysis of the four E2 derivatives required protein expression, facilitated by placing the E2 genes on a high copy plasmid behind the *CUP1* promoter. A detailed schematic of a high copy *CUP1* overexpression vector is provided in Figure 2.1. *Kpn*I-*Sst*I digested PCR products of the *Arg49 UBC4*, *Ser62 UBC4* and *Asp95 UBC4* genes were introduced into the *URA3* vector in place of the Ub cassette. These restriction sites were introduced to the DNA sequences outside of the *UBC4* or *RAD6Δ* coding regions by the PCR primers during construction. The *Rad6Δ* mutant was transferred from the high copy *TRP1* vector to the *URA3* based vector as a *Kpn*I-*Sst*I fragment.

**Phenotype Analysis**

**a. Canavanine sensitivity analysis.**

The yeast strain deleted for the *UBC4* and *UBC5* genes (*ubc4/5Δ*) was transformed individually with each of the low copy number plasmids expressing the three *UBC4*

mutants and tested for viability in the presence of canavanine. Cell survival in the presence of canavanine was determined by plating exponentially growing yeast cells on minimal media plates supplemented with lysine (40 mg/liter) and uracil (2.4 mg/liter) and also containing 1.7 μg canavanine per ml. Copper was not included, as the *UBC4* mutant genes are under the control of their own promoter. The UBC4 mutant plasmids were maintained by tryptophan selection. Colonies were counted after 6 days of incubation at 30°C. $2x10^4$, $2x10^3$, and $2x10^2$ cells were plated for each strain in duplicate. Percent survival was expressed as survival in the presence of canavanine compared to the same dilutions of the same strain plated in the absence of canavanine.

The RAD6/UBC4 chimaera was expressed in the *ubc4/5Δ* strain and tested in an identical manner as for the low copy UBC4 mutants with one exception. Unlike the UBC4 mutants, copper was included in the medium to induce protein expression from the *CUP1* promoter.

**b. Chronic heat stress analysis**

The *UBC4/5* deleted yeast strain was tested for stress sensitivity when producing each of the three UBC4 derivatives individually, as well as the RAD6/UBC4 chimaera. Cell survival after chronic heat stress was determined as for canavanine survival, except that canavanine was omitted from the medium, and plates were incubated at 39°C for exactly 24 hours, followed by a shift to 30°C for 4 days to allow for colony development. The UBC4 mutant plasmids and the RAD6Δ mutant plasmid were maintained by tryptophan selection. As for the canavanine analysis, each strain was plated in duplicate for both the stress and unstressed experiments. Percent survival was expressed as the survival after 24 hours of heat stress as compared to the survival of the same dilution of each strain in the absence of heat stress. Copper was again included only for the RAD6/UBC4 chimaera.

**c. Growth analysis.**

Cellular doubling time was calculated from the growth rate over an 8 hour time course. *UBC4/5* deleted yeast cells transformed with the RAD6/UBC4 chimaera, the UBC4 mutants Ser62, Arg49 or Asp95, wild type UBC4 or no gene, were grown in 25 ml tryptophan dropout medium in liquid culture at 30°C. When the cultures reached mid-log phase (~2 x $10^7$ cells/ml), small aliquots of each culture were taken at predetermined time points and the number of cells per ml was determined using a Coulter Counter. For each time point, the culture was diluted 10 fold in Isoton (essentially phosphate buffered saline) with 3% formaldehyde to arrest cell growth, in a 2 ml volume. Before counting the cells,

the diluted samples were vortexed, diluted another 20 fold, and sonicated for 6 seconds to disrupt any cellular aggregation. The readings from the Coulter counter were taken in duplicate. The growth rate was reported as the time required for a given yeast strain to double the number of cells per ml at 30°C in SD medium lacking only tryptophan.

**Alternative linkage formation of the UBC4 mutants and the RAD6/UBC4 chimaera**

Analysis of the Ub-Ub linkages formed by the UBC4 mutants and the RAD6/UBC4 chimaera was determined in a manner identical to previous experiments (Chapter 3). The *UBC4/5* deleted yeast strain was doubly transformed with both a *URA3* and *TRP1* plasmid. The *TRP1* plasmids expressed the Ubm derivatives whose use and construction have been previously described (Appendix A). The *URA3* based vectors, however, produced one of the UBC4 point mutants or the RAD6/UBC4 chimaera from the *CUP1* promoter (see below for construction). *CUP1* overexpression, sample preparation, and anti Myc Western analysis have been described in detail in Appendix A.

## 4.3 Results

### 4.3.1 The E2 derivatives.

The amino acid changes leading to CDC34 (UBC3) temperature sensitive function which were used to create a UBC4 *ts* are shown in Figure 4.1, indicating that these three residues are present at the corresponding positions in UBC4. A three dimensional representation of the yeast UBC4 protein structure (Cook *et al.*, 1993; Figure 4.2) is shown to demonstrate that the three amino acid mutations are surface residues. Serine 95 is spatially adjacent to the Proline 62 residue and this residue is also flanked by two conserved residues, tryptophan (W) and proline (P) on either side in a WSP motif common to most E2s (Figure 4.1). A second point to note are the changes introduced into the RAD6Δ derivative. Specifically, the two amino acids conserved in all UBC4 homologs (Phe63 and Asn80) have replaced the corresponding amino acids which in turn are conserved in all RAD6 homologs (Figure 4.1), and are likewise surface-exposed amino acid residues (Figure 4.2b; Cook *et al.*, 1992). Secondly, the 3D structure of UBC4 and a RAD6Δ homolog are highly conserved, and are almost superimposeable.

### 4.3.2. Growth analysis of E2 mutants.

The *ubc4/5Δ* strain is temperature stressed even at temperatures as low as 30°C. However, at 30°C the cell proliferation rate is merely slowed and no viability is lost

(unpublished observation). At higher temperatures, the *ubc4/5Δ* strain is increasingly growth defective, and becomes inviable even after transfer to permissive temperatures (Seufert and Jentsch, 1990). Expression of wild type UBC4 in this strain allows for growth at temperatures tested up to 40°C, inferring that UBC4 function is required for growth at higher temperatures.

Plasmids expressing either the wild type UBC4 protein, the Arg49, the Ser62, the Asp95 UBC4 mutants, no gene, wild type RAD6 or the RAD6/UBC4 chimaera were introduced into a yeast strain deleted for the endogenous *UBC4/5* genes (*ubc4/5Δ*) . These strains were then tested for their ability to grow at different temperatures. At the permissive temperature of 30°C, (Figure 4.3a and 4.4a) the growth rate of wild type UBC4, Ser62 UBC4 and Arg49 UBC4 was indistinguishable, based on their similar colony size. The colonies formed by the *ubc4/5Δ* strain (null) were significantly smaller, in agreement with the cell proliferation defect associated with the loss of these two genes (Seufert and Jentsch, 1990). As expected, expression of RAD6 did not increase colony size above that of the null strain, as RAD6 cannot complement for UBC4/5 function. In contrast, the Asp95 UBC4 and RAD6/UBC4 chimaera mutants formed colonies intermediate in size between the wild type and *UBC4/5* deleted yeast strain.

For the UBC4 derivatives at the nonpermissive temperature of 39°C, however (Figure 4.3b), it was found that only the null *ubc4/5Δ* strain was completely inviable. Each of the three UBC4 point mutants was capable of some growth at 39°C. The Arg49 UBC4 mutant appeared to grow at a rate comparable to that observed for the *ubc4/5Δ* strain expressing wild type UBC4. In contrast to this, both the Asp95 and Ser62 UBC4 mutants were significantly impaired for cell growth. For the RAD6/UBC4 chimaera and RAD6, it was determined that at 35.5°C, neither was able to grow and exhibited the same proliferation defect as the *ubc4/5Δ* strain (Figure 4.4b). This indicated that the RAD6/UBC4 chimaera was strongly temperature sensitive for growth at 35.5°C. These results demonstrate that the four E2 derivatives used differ in their temperature sensitivity, and that some were unaffected for function at high temperatures.

Quantitative analysis of growth rates at the permissive temperature by cell counting yielded results that were consistent with the qualitative growth assessment described above (Table 4.1A). The vegetative (logarithmic) growth rate of *ubc4/5Δ* cells producing wild type UBC4, Ser62 UBC4 and Arg49 UBC4 at 30°C were similar and ranged from 1.69-2.11 hr per doubling period. The *ubc4/5Δ* strain, as expected, had an increased doubling time, (7.8 hr) consistent with previously reported doubling times (Seufert and Jentsch, 1990). The Asp95 UBC4 and RAD6/UBC4 derivatives had doubling times intermediate between wild type UBC4 and the null strain, calculated to be 4.15 hr and 3.85 hr,

respectively, demonstrating that the Asp95 UBC4 derivative was partially defective in providing UBC4-dependent growth, and interestingly, that the RAD6/UBC4 derivative had gained partial ability to complement for UBC4-dependent growth.

### 4.3.3. Sensitivity of E2 derivatives to chronic heat stress.

To see if the four E2 derivatives also exhibited defects in other aspects of UBC4 function, the derivatives were investigated for their abilities to complement for the loss of UBC4/5 function in stress resistance. We first tested heat stress sensitivity which, in contrast to the growth rate measurements described in the previous section, is traditionally measured by plating experiments that measure cell survival by colony formation at the permissive temperature following exposure to heat stress.

*Ubc4/5Δ* strains, expressing each of the RAD6Δ or UBC4 mutants were exposed to 39°C for 24 hours to stress the cells, followed by transfer to 30°C to allow cells to recover and colonies to develop. As seen in Table 4.1B, only the Ser62 UBC4 derivative exhibited marked sensitivity to chronic heat stress with a reduction in viability that was equal to that of the ubc4/5Δ strain (4% survival). The RAD6/UBC4 derivative exhibited modest sensitivity to heat stress (25% survival) while the Asp95 and Arg49 UBC4 derivative exhibited sensitivities close to wild type UBC4 (66%, 78% and 83%, respectively).

### 4.3.4. Sensitivity of E2 derivatives to canavanine.

Another stress to which the *ubc4/5Δ* strain is extremely sensitive is growth in the presence of the amino acid analog canavanine. *ubc4/5Δ* cells expressing the three UBC4 mutants were tested for their ability to confer resistance to these cells when grown in the presence of canavanine. As shown in Table 4.1C, the RAD6/UBC4 exhibited the greatest sensitivity to canavanine stress with a reduction in viability that was equal to that of the *ubc4/5Δ* strain (<0.02% survival). Wild type stress resistance was diminished in two of the UBC4 mutants, the Asp95 UBC4 mutant and the Ser62 UBC4 mutant (31% and 51% resistance of wild type, respectively). In contrast, the Arg49 UBC4 mutant exhibited little change in resistance to canavanine from wild type (69% versus 77%).

### 4.3.5. The efficiency of the E2 derivatives at forming the alternative Ub-Ub linkages.

In Chapter 3 we established that UBC4/5 participated in the conjugation of Ub to Ub at lysines (K) 29 and 63 and that the K63 linkage played an important role in the yeast stress response. In view of phenotypic difference exhibited by the UBC4 and

RAD6/UBC4 mutants described thus far in response to stress, it was of interest to determine if differences also existed in their ability to make alternative Ub-Ub linkages.

The effectiveness of these mutants in alternative linkage formation was examined as described in Chapter 3. Briefly, each UBC4 and RAD6 mutant plasmid was introduced into the *ubc4/5Δ* deletion strain in combination with plasmids expressing each of three Ub derivatives which distinguish, *in vivo*, between the creation of Ub-Ub conjugates formed at positions 29 or 63. Detection of Ub-Ub conjugates was facilitated by Western analysis using the anti Myc antibody as a probe. The results of these experiments are shown in Figure 4.5 and indicate that the Ser62 UBC4 and Asp95 UBC4 derivatives are capable of forming both the K29 and K63 Ub-Ub linkages. Surprisingly, the RAD6/UBC4 derivative was also observed to be capable of creating the K29 and K63 Ub-Ub conjugate, in contrast to wild type RAD6 which can form only the K63 Ub-Ub conjugate (Chapter 3, Figure 3.4).

Based on this experiment it is clear that although there is a reduction in K29 and K63 linkage formation relative to wild type, all mutants tested have the capacity to make K29 and K63 Ub-Ub conjugates *in vivo*. Thus, the canavanine sensitivity observed for the Asp95 UBC4 derivative and the RAD6/UBC4 chimaera is not due to a defect in alternative linkage assembly, but some other aspect of E2 function.

## 4.4 Discussion

Four derivatives of UBC4 were created by altering surface accessible amino acids in order to further elucidate the function of UBC4 in either heat or amino acid analog stress resistance, and in cellular growth. These E2 derivatives were used to elucidate the biological and biochemical functions of UBC4, and secondly, were analyzed for their utility in a genetic screen for UBC4 interacting proteins.

For the UBC4 derivatives to be useful in a genetic screen, there were two requirements. First, the mutants must have a sufficiently low nonpermissive temperature at which growth differences between wild type and mutant E2s is dramatic. Secondly, such mutants must have low reversion frequencies such that the number of false positives is negligible. The Ser62 UBC4 derivative was found to be unsuitable because of a high reversion frequency, similar to that observed in the *ts* CDC34 protein carrying the corresponding mutation (J. Prendergast, unpublished observation). The Arg49 UBC4 derivative was discarded because it was not temperature sensitive. The Asp95 UBC4 derivative was interesting for reasons discussed below, but was not suitably temperature sensitive for the proposed screen. Lastly, the RAD6/UBC4 chimaera was found to be *ts*,

and was considered useful for the screen. The utility of the RAD6/UBC4 chimaera in the genetic screen for interacting proteins is discussed in the following chapter.

Of the four E2 derivatives examined here, the behavior of two strongly suggests that the function of UBC4 in growth, canavanine resistance, and heat stress resistance varies from one to another. The defect of Asp95 UBC4 with respect to growth and canavanine resistance, but not heat stress resistance, suggests that the role of UBC4 in the former two functions differs from the latter. Additionally, the RAD6/UBC4 chimaera gains the ability to carry out UBC4 specific functions upon the introduction of two UBC4-specific amino acids. The RAD6/UBC4 chimaera partially complemented for UBC4 function in both cellular proliferation and heat stress resistance (whereas the corresponding RAD6 protein does not, Figure 4.4), but is unable to complement for resistance to canavanine (Table 4.1).

These results suggest two hypotheses pertaining to UBC4 biochemical function in growth, heat and amino acid analog resistance. First, it may be that the different UBC4 functions require the turnover of specific, but independent protein targets for each function. Therefore, it may be that the different aspects of UBC4 function do not require the turnover of the general class of damaged or abnormal proteins, but requires the selective targeting of specific subclasses for each. Secondly, it may be that UBC4 does function by the degradation of nonspecific misfolded proteins, but that the recognition of canavanyl, thermally denatured, or growth related targets are facilitated by three different associated factors which respond differently to the various UBC4 mutations. Following this line of reasoning, the RAD6/UBC4 chimaera would be completely unable to interact with the canavanine specific *trans*-acting factor(s), whereas the Asp95 UBC4 mutant would be able to partially associate, reflecting their different degrees of functional loss for canavanine resistance. In turn, the Asp95 UBC4 mutant would be able to fully interact with the heat stress specific *trans*-acting factors(s) to provide wild type resistance, whereas the RAD6/UBC4 chimaera could not, explaining the inability to fully complement for heat stress resistance. The juxtaposition of these results from the two E2 derivatives suggests that the growth, heat, and canavanine associated functions of UBC4 are not part of the same cellular pathway.

Although the Ser62 UBC4 derivative also appeared to fit this pattern, the differences of phenotypic behavior may be attributed to more trivial reasons. Specifically, the marked heat stress sensitivity of this derivative is likely the result of structural instability at higher temperatures, a notion supported by the observation that the altered proline residue in this derivative is part of a conserved turn motif (Ellison *et al.*, 1991). The maintenance of growth and (significant) canavanine resistance functions at permissive

temperatures further indicates that the Ser62 UBC4 derivative is truly a temperature sensitive UBC4 derivative.

Surprisingly, the canavanine sensitive and heat stress sensitive E2 derivatives were capable of forming both the K29 and K63 Ub-Ub conjugates. Therefore, the loss/absence of stress resistance function of the E2 derivatives was not correlated with the loss of the stress-related K63 Ub-Ub conjugate (Chapter 3). This implies that the presence of this linkage was not sufficient for function but instead must be targeted to specific proteins, a function which has been lost or decreased in the Asp95 UBC4 and RAD6 mutants. Remarkably, the substitution of just two residues in RAD6 to their UBC4 counterpart results in an E2 derivative that acquires the ability to make the K29 Ub-Ub linkage. Two observations suggest that the K29 Ub-Ub conjugate may have a role in cellular growth. First, the absence of the K29 linkage by replacement with arginine results in a slowed growth rate when the Ub derivative is the sole source of cellular Ub (Finley *et al.*, 1994). Secondly, the gained ability of the RAD6/UBC4 chimaera to create this linkage is correlated with the ability to partially complement for the growth rate deficiency observed in the *UBC4/5* deletion strain.

From a structure/function perspective, the mutant analysis presented in this chapter represents our first attempts to correlate different aspects of UBC4 function with defined areas of the UBC4 protein surface. Together, these results suggest that UBC4 amino acids Ser95, Phe63 and Asn80 are involved in cellular growth and/or heat stress resistance function. It may be that the different UBC4 functions are dependent on different protein associations, and that the aforementioned surface accessible amino acid residues on UBC4 are important for these associations. In the next chapter, the RAD6/UBC4 chimaera, which has the Phe63 and Asn80 residues introduced at the corresponding positions in RAD6, is used in a genetic screen to identify genes whose protein products are candidates for being UBC4-interacting factors.

FIGURE 4.1

**Protein Sequence alignment of ubiquitin-conjugating enzymes.**

Residues of interest (see text) are shown in ***boldface***. Dots denote gaps in the protein sequence alignment. The sequences encoding for UBC4 and RAD6 (UBC2) proteins are grouped together with their respective homologs from different eukaryotes, as indicated below. The additional sequences are a selection of other Ub-conjugating enzymes identified in *S. cerevisiae* with intracellular functions distinct from either RAD6 or UBC4. The protein sequence alignment was obtained using Ig Suite.

The three point mutations introduce into the UBC4 protein sequence are represented as follows: ♦, the glycine to arginine conversion at UBC4 residue number 49 (Arg49 UBC4). ♣, the proline to serine conversion at UBC4 residue number 62 (Ser62 UBC4). ♠, the serine to aspartate conversion at UBC4 residue number 95 (Asp95 UBC4). The relevant mutations introduced into the RAD6 protein sequence to generate the RAD6/UBC4 chimera (RAD6Δ Phe65 Asn82) are represented as follows: •, the asparagine to phenylalanine conversion at RAD6 residue number 65. *, the tyrosine to asparagine conversion at RAD6 residue number 82.

Those UBC sequences terminating with Y or no letter (left hand column) represent sequences from *S. cerevisiae*. The remaining sequences are from the following organisms: UBC4C: UBC4 homolog from *C. elegans*. UBC4D: UBC4 homolog from *Drosophila*. UBC4H: one of two UBC4 homologs from human. UBC2HA and UBC2HB: two RAD6 (UBC2) homologs from human. UBC2D: RAD6 homolog from *Drosophila*. UBC2A: RAD6 homolog from *Arabadopsis*. Note that the *S. cerevisiae* UBC1 C-terminal sequence has been truncated.

```
UBC4Y  .....MSSSK RIAKELSDLE RDPPTSCS.. AGPVGD.DLY HWQASI.MGP
 UBC5  .....MSSSK RIAKELSDLG RDPPASCS.. AGPVGD.DLY HWQASI.MGP
UBC4C  ......MALK RIQKELQDLG REPPAQCS.. AGPVGD.DLF HWQATI.MGP
UBC4D  ......MALK RINKELQDLG REPPAQCS.. AGPVGD.DLF HWQATI.MGP
UBC4H  ......MALK RIHKELNDLA RDPPAQCS.. AGPVGD.DMF HWQATI.MGP
 UBC1  .....MSRAK RIMKEIQAVK DDPAAHIT.. LEFVSESDIH HLKGTF.LGP
UBC2HA ...MSTPARR RLMRDFKRLQ EDPPAGVS.. GAP.SENNIM VWNAVI.FGP
UBC2HB ...MSTPARR RLMRDFKRLQ EDPPVGVS.. GAP.SENNIM QWNAVI.FGP
 UBC2D ...MSTPARR RLMRDFKRLQ EDPPTGVS.. GAP.TDNNIM IWNAVI.FGP
 UBC2A ...MSTPARK RLMRDFKRLQ QDPPAGIS.. GAP.QDNNIM LWNAVI.FGP
  UBC2 ...MSTPARR RLMRDFKRMK EDAPPGVS.. ASP.LPDNVM VWNAMI.IGP
  UBC7 ...MSKTAQK RLLKELQQLI KDSPPGIV.. AGPKSENNIF IWDCLI.QGP
  UBC8 ......... .....MKLLM SDHQVDLI.. NDSMQE.... .FHVKF.LGP
  UBC3 MSSRKSTASS LLLRQYRELT DPKKAIPSFH IELEDDSNIF TWNIGVNVLN

                ♦                ♣•               *
UBC4Y  ADSPYAGGVF FLSIHFPTDY PFKPPKISFT TKIYHPNINA .NGNICLDIL
 UBC5  SDSPYAGGVF FLSIHFPTDY PFKPPKVNFT TKIYHPNINS .SGNICLDIL
UBC4C  PESPYQGGVF FLTIHFPTDY PFKPPKVAFT TRIYHPNINS .NGSICLDIL
UBC4D  PDSPYQGGVF FLTIHFPTDY PFKPPKVAFT TRIYHPNINS .NGSICLDIL
UBC4H  NDSPYQGGVF FLTIHFPTDY PFKPPKVAFT TRIYHPNINS .NGSICLDIL
 UBC1  PGTPYEGGKF VVDIEVPMEY PFKPPKMQFD TKVYHPNISS VTGAICLDIL
UBC2HA EGTPFGDGTF KLTIEFTEEY PNKPPTVRFV SKMFHPNVYA .DGSICLDIL
UBC2HB EGTPFEDGTF KLVIEFSEEY PNKPPTVRFL SKMFHPNVYA .DGSICLDIL
 UBC2D HDTPFEDGTF KLTIEFTEEY PNKPPTVRFV SKVFHPNVYA .DGGICLDIL
 UBC2A DDTPWDGGTF KLSLQFSEDY PNKPPTVRFV SRMFHPNIYA .DGSICLDIL
  UBC2 ADTPYEDGTK RLLLEFDEEY PNKPPHVKFL SEMFHPNVYA .NGEICLDIL
  UBC7 PDTPYADGVF NAKLEFPKDY PLSPPKLTFT PSILHPNIYP .NGEVCISIL
  UBC8 KDTPYENGVW RLHVELPDNY PYKSPSIGFV NKIFHPNIDI ASGSICLDVI
  UBC3 EDSIYHGGFF KAQMRFPEDF PFSPPQFRFT PAIYHPNVY. RDGRLCISIL

                                                      ♣
UBC4Y  .......... ...KDQWSPA LTLSKVLLS. ICSLLTDANP DDPLVPEIAH
 UBC5  .......... ...KDQWSPA LTLSKVLLS. ICSLLTDANP DDPLVPEIAQ
UBC4C  .......... ...RSQWSPA LTLSKVLLS. ICSLLCDPNP DDPLVPEIAR
UBC4D  .......... ...KSQWSPA LTLSKVLLS. ICSLLCDPNP DDPLVPEIAR
UBC4H  .......... ...RSQWSPA LTISKVLLS. ICSLLCDPNP DDPLVPEIAR
 UBC1  .......... ...KNAWSPV ITLKSALIS. LQALLQSPEP NDPQDAEVAQ
UBC2HA .......... ...QNRWSPT YDVSSILTS. IQSLLDEPNP NSPANSQAAQ
UBC2HB .......... ...QNRWSPT YDVSSILTS. IQSLLDEPNP NSPANSQAAQ
 UBC2D .......... ...QNRWSPR YDVSAILTS. IQSLLSDPNP NSPANSTAAQ
 UBC2A .......... ...QNQWSPI YDVAAILTS. IQSLLCDPNP NSPANSEAAR
  UBC2 .......... ...QNRWTPT YDVASILTS. IQSLFNDPNP ASPANVEAAT
  UBC7 HSPGDDPNMY ELAEERWSPV QSVEKILLS. VMSMLSEPNI ESGANIDACI
  UBC8 .......... ...NSTWSPL YDLINIVEWM IPGLLKEPNG SDPLNNEAAT
  UBC3 HQSG.DPNTD EPDAETWSPV QTVESVLIS. IVSLLEDPNI NSPANVDAAV


UBC4Y  ..IYKTDRPK YEATAREWTK KYAV..... .......... ..........
 UBC5  ..IYKTDKAK YEATAKEWTK KYAV..... .......... ..........
UBC4C  ..IYKTDRER YNQLAREWTQ KYAM..... .......... ..........
UBC4D  ..IYKTDREK YNELAREWTR KYAM..... .......... ..........
UBC4H  ..IYQTDREK YNRIAREWTQ KYAM..... .......... ..........
 UBC1  ..HYLRDRES FNKTAALWTR LYASETSNGQ KGNVEESDLY GIDHDLIDEF
UBC2HA ..LYQENKRE YEKRVSAIVE QSWRDC.... .......... ..........
UBC2HB ..LYQENKRE YEKRVSAIVE QSWNDS.... .......... ..........
 UBC2D ..LYKENRRE YEKRVKACVE QSFID..... .......... ..........
 UBC2A ..MYSESKRE YNRRVRDVVE QSWTAD.... .......... ..........
  UBC2 ..LFKDHKSQ YVKRVKETVE KSWE...... .......... ..........
  UBC7 ..LWRDNRPE FERQVKLSIL KSLGF..... .......... ..........
  UBC8 ..LQLRDKKL YEEKIKEYID KYATKEKYQQ MFGGD..... ..........
  UBC3 ..DYRKNPEQ YKQRVKMEVE RSKQ...... .......... ..........
```

FIGURE 4.2

**Stereo three-dimensional protein structures of *S. cerevisiae* UBC4 and the *Arabadopsis* RAD6 homolog.**

Shown are the stereo three dimensional images of the yeast UBC4 protein and the RAD6 homolog from *Arabadopsis*. The N-terminus of each protein is indicated by **N**. The protein backbone is represented by gray lines. Relevant amino acids side chains are in black. The image of the RAD6 homolog is rotated horizontally 180° to that of the UBC4 structure in order to best display the relevant amino acid residues.

*S. cerevisiae* UBC4. The three amino acid residues individually altered in the UBC4 mutants are indicated as is the active site cysteine (C86). The glycine 49 (Gly49) residue was converted to arginine to create the Arg49 UBC4 mutant. The proline 62 (Pro62) residue was converted to serine (Ser62 UBC4). The serine 95 (Ser5) residue was converted to aspartic acid (Asp95 UBC4).

*Arabadopsis* RAD6 homolog. The Arabadopsis RAD6 homolog lacks the polyacidic tail of the yeast RAD6 protein. The RAD6 mutant used in Chapter 4 had the C-terminal 23 amino acids deleted, and therefore, this crystal structure is a fair representation of the RAD6Δ mutant. Also indicted is the active site cysteine (C88) and the two amino acids which were altered. The asparagine 65 (Asn65) residue was converted to phenylalanine (Phe65) and the tyrosine 82 (Tyr82) residue was converted to asparagine (Asn82). These figures were constructed using the Power MacImdad Interactive Molecular Display and Design software.

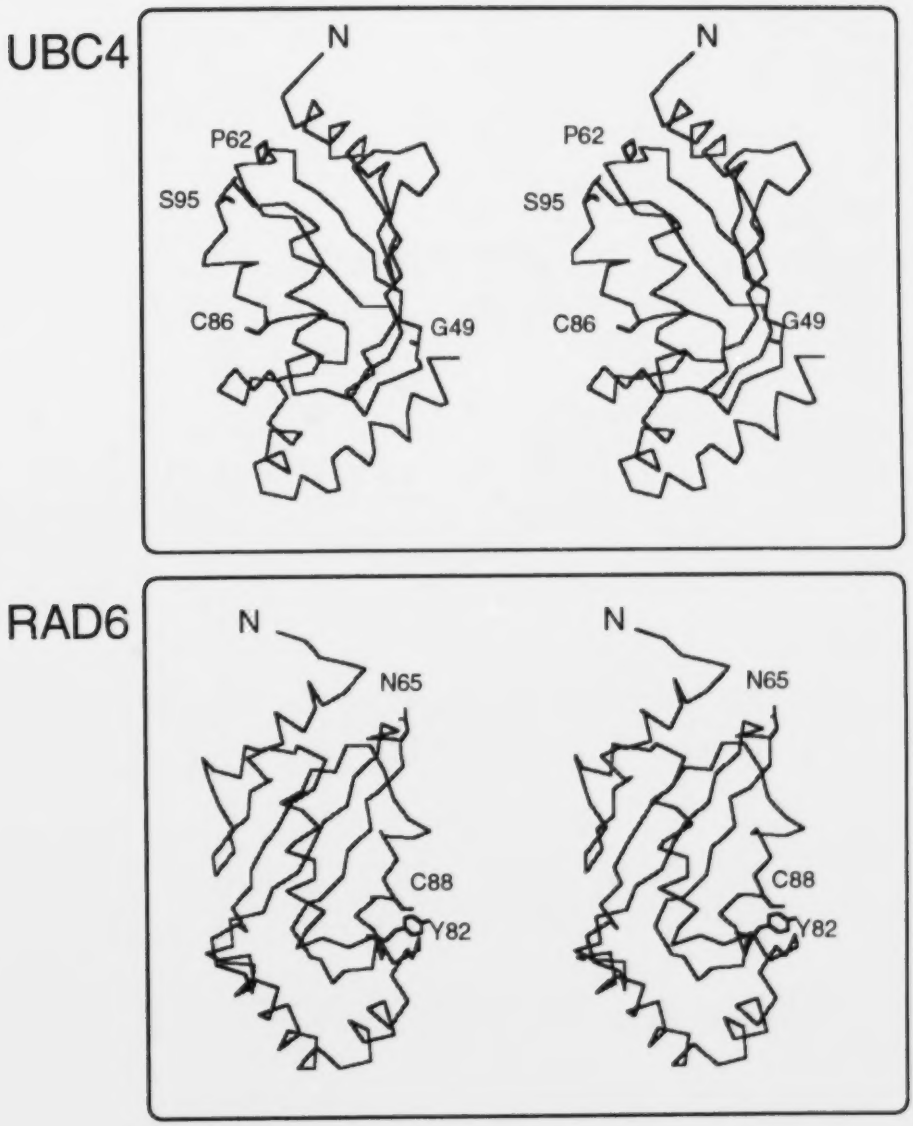
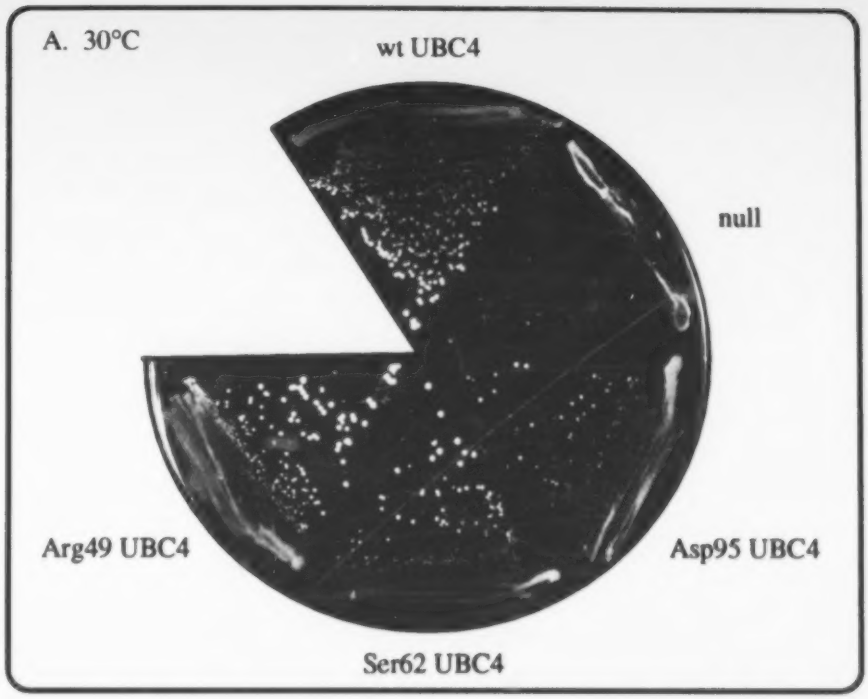

UBC4
N
P62
S95
C86
G49
N
P62
S95
C86
G49
RAD6
N
N65
C88
Y82
N
N65
C88
Y82

FIGURE 4.3

**Three UBC4 point mutants have variable temperature sensitivities in a *S. cerevisiae UBC4/5* deletion strain.**

The *UBC4/5* deletion strain was transformed with a series of low copy plasmids expressing wild type UBC4 (wt), no gene (null), or one of three UBC4 point mutants (Arg49 UBC4, Ser62 UBC4, or Asp95 UBC4). A single colony of each transformed yeast strain was streaked onto SD medium enriched with all amino acids but tryptophan, for plasmid selection. The plates were incubated at 30°C for 4 days (Part A) or 39°C (Part B) for 6 days.


A. 30°C
wt UBC4
null
Arg49 UBC4
Asp95 UBC4
Ser62 UBC4

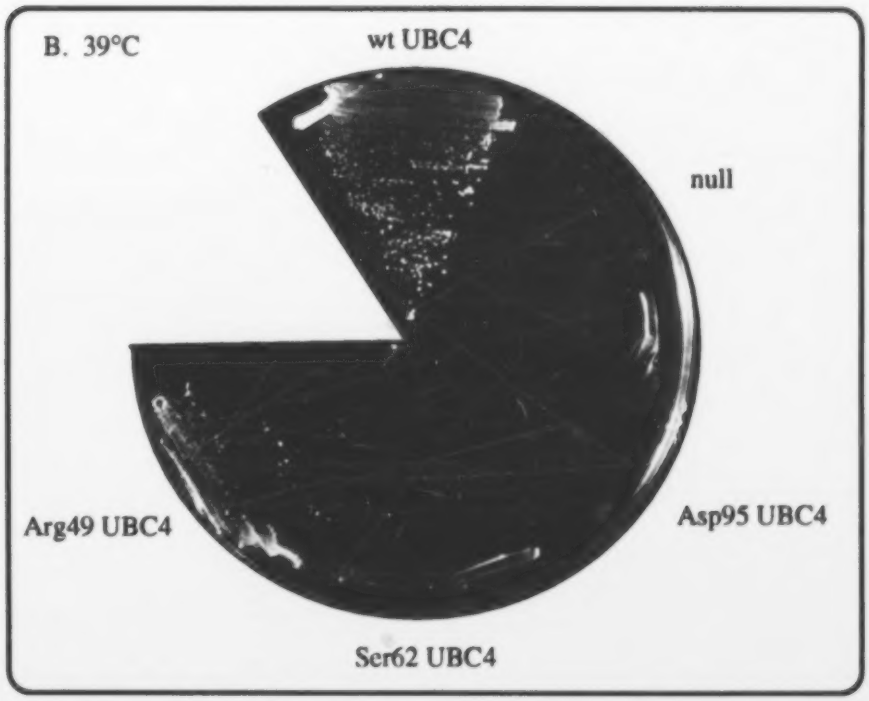
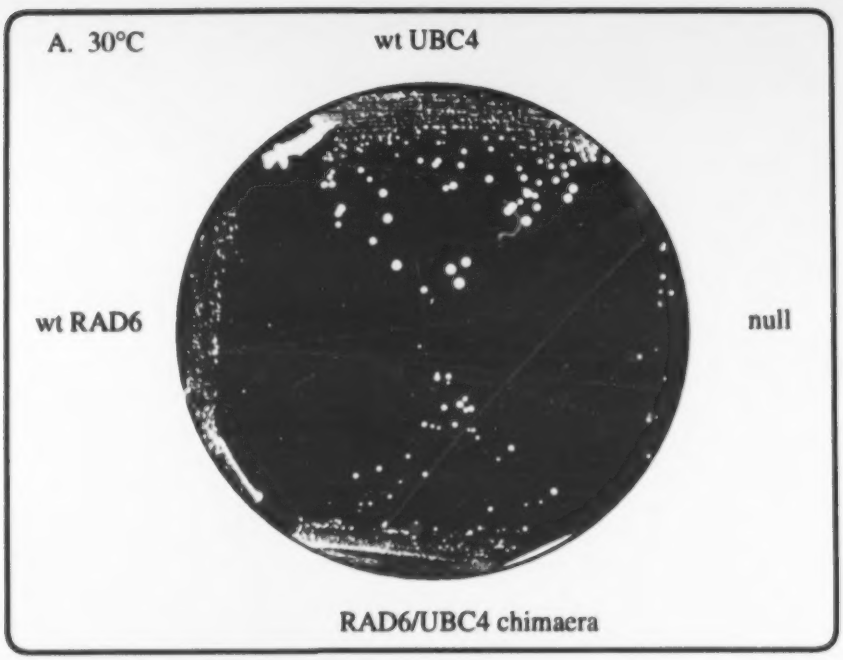

B. 39°C
wt UBC4
null
Arg49 UBC4
Asp95 UBC4
Ser62 UBC4

FIGURE 4.4

**A RAD6/UBC4 chimaeric protein is temperature sensitive and partially complements for the cell proliferation defect in a *S. cerevisiae UBC4/5* deletion strain.**

The yeast *UBC4/5* deletion strain was transformed with a series of high copy plasmids expressing wild type *UBC4* (wt), no gene (null), wild type *RAD6* , or the RAD6/UBC4 chimaera. A single colony of each transformed yeast strain was streaked onto SD medium containing 100 μM $CuSO_4$ for *CUP1* promoter induction, and enriched with all amino acids but tryptophan, for plasmid selection. The plates were incubated at 30°C for 4 days (Part A) or 35.5°C (Part B) for 6 days.


A. 30°C
wt UBC4
wt RAD6
null
RAD6/UBC4 chimaera

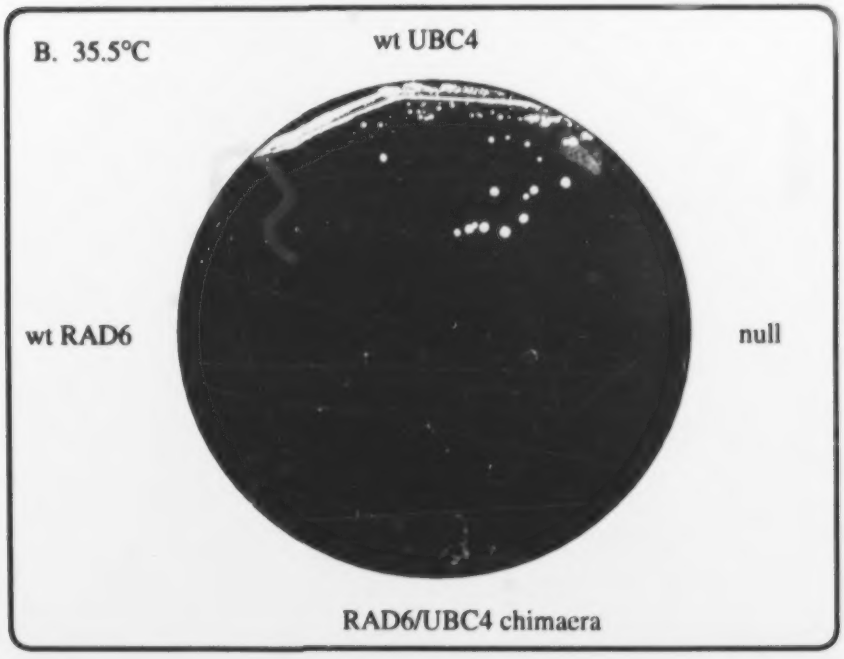
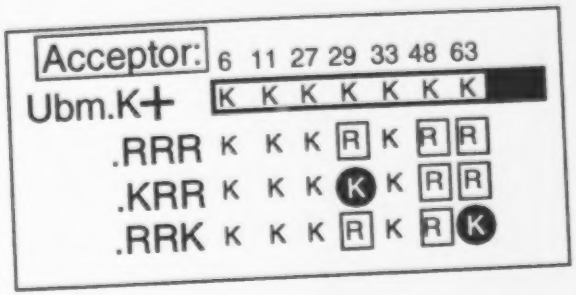

B. 35.5°C
wt UBC4
wt RAD6
null
RAD6/UBC4 chimaera

FIGURE 4.5

**UBC4 and RAD6 derivatives create both K29 and K63 Ub-Ub conjugates when overexpressed in the *ubc4/5Δ* strain.**

Shown is an SDS PAGE anti-Myc Western blot of total protein from yeast cells expressing C-terminal tagged Ub acceptors (Ubm). For Ubm derivatives, only lysines identified to be targets of ubiquitination (circled positions) have been mutated to arginine in the combinations shown (boxed positions). Ubm derivatives are expressed in cells that are deleted for the *UBC4* and *UBC5* genes (*ubc4/5Δ*, Part a), or in combination with the Ser62 UBC4 mutant (Part b), Asp95 UBC4 mutant (Part c), or the RAD6/UBC4 chimaera (Part d). The E2 derivatives are expressed from high copy number plasmids. Numbers mark the position of lysines (K) present in the wild type Ub sequence (Ubm.K+). The Myc epitope is shown as a black box. For gels, the position of monomeric and dimeric Ubm and Ubm conjugates are as indicated. Part a is a duplicate of Figure 3.1 b, and is included as a reference. Also indicated are the positions of molecular weight marker standards. *Gel lanes:* +, O, 29 and 63 refer to the available lysines in the Ubm derivatives K+, RRR, KRR, and RRK, respectively.


Acceptor: 6 11 27 29 33 48 63
Ubm.K+ K K K K K K K
.RRR K K K R K R R
.KRR K K K K K R R
.RRK K K K R K R K

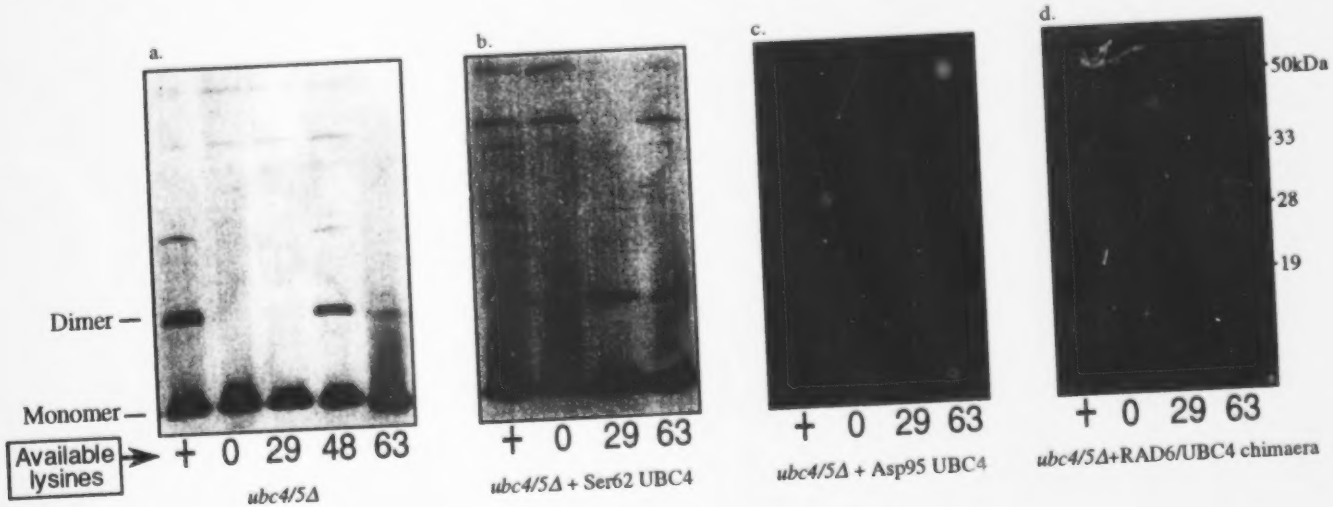

a.
b.
c.
d.
Dimer
Monomer
Available lysines
+ 0 29 48 63
+ 0 29 63
+ 0 29 63
+ 0 29 63
50kDa
33
28
19
ubc4/5Δ
ubc4/5Δ + Ser62 UBC4
ubc4/5Δ + Asp95 UBC4
ubc4/5Δ+RAD6/UBC4 chimaera

TABLE 4.1

*UBC4/5* deletion strain growth and stress characteristics when expressing *UBC4* and *RAD6* derivatives.

| strain | Vegetative growth (doubling time in h) | % Survival Heat | % Survival Canavanine |
|---|---|---|---|
| | A | B | C |
| *ubc4/5Δ* | 7.8 | 4 | <0.02 |
| *ubc4/5Δ +UBC4* | 1.69 | 83 | 77 |
| *ubc4/5Δ +Arg49 UBC4* | 1.77 | 78 | 69 |
| *ubc4/5Δ +Ser62 UBC4* | 2.11 | 4 | 40 |
| *ubc4/5Δ +Asp95 UBC4* | 4.15 | 68 | 24 |
| *ubc4/5Δ +RAD6/UBC4* | 3.85 | 24 | <0.02 |

Shown are the calculated growth and survival rates of yeast cells deleted for the *UBC4* and *UBC5* genes when expressing various derivatives of the UBC4 and RAD6 proteins. *UBC4*, *Arg49 UBC4*, *Ser62 UBC4*, and *Asp95 UBC4* indicates the presence of the UBC4 or UBC4 derivatives expressed from a low copy number plasmid. The RAD6/UBC4 chimaera is expressed from a high copy number plasmid. **A**. Calculation of the growth rates of cells grown in SD media at 30°C. Doubling times were determined by counting equal aliquots of cells from each culture using a cell counter (see Materials and Methods). **B**. % survival of yeast cells after exposure to heat stress was determined by plating assays under the conditions described in Materials and Methods. Values represent the percentage of survivors relative to that of the corresponding unstressed cells. **C**. % survival of yeast cells grown in the presence of canavanine, as outlined in Materials and Methods. Values represent the percent survivors relative to unstressed cells.

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# CHAPTER 5. A Genetic Strategy to Identify Genes Related to UBC4 Function.

## 5.1 Introduction

The discovery of alternative lysine residues available for Ub-Ub conjugation (Chapter 2), and the identification of the E2s responsible for their formation (Chapter 3), added a layer of complexity to the understanding of the process of Ub-protein targeting. It is generally believed that the direct interaction of E2 proteins with associated factors, including E3s, *trans*-acting factors, and other E2s, contributes to target specificity (reviewed in Ciechanover and Schwartz, 1994). It may also be, therefore, that the interaction of an E3, or associated factor, provides the guidelines that determine linkage specificity.

In the case of UBC4, the relevant *in vivo* targets, as well as the mechanism and cofactors that govern UBC4 substrate selectivity, are largely unknown. In chapter 4 it was shown that specific amino acids on the surface of UBC4, (Ser95, Phe63 and Asn80) apparently affect different UBC4 functions. Therefore, it may be that these residues constitute portions of recognition sites for *trans*-acting protein factors necessary for these UBC4 functions.

In this study a genetic screen was employed to identify genes that encode proteins that could interact with UBC4. The rationale of this screen is based on the idea that overexpression of such a protein from a high copy plasmid would favor the E2-protein interaction, thereby relieving the thermal lability of the E2 and resulting in an increased resistance of the mutant to elevated temperature (see Prendergast *et al.*, 1995 for example). To identify proteins which directly interact with the UBC4 protein, a yeast genomic overexpression library (Carlson and Botstein, 1982) was screened. A mutant capable of temperature sensitive UBC4 function was available (the RAD6/UBC4 chimaera, Chapter 4) for use in this screen. The return of UBC4 function at the nonpermissive temperature would therefore allow for cellular growth at temperatures where previously no growth was observed. This chapter describes the selection, isolation, and analysis of a yeast gene capable of conferring growth at the nonpermissive temperature to a *ts S. cerevisiae* strain specifically defective in UBC4 function at nonpermissive temperatures.

## 5.2 Materials and Methods

### *Saccharomyces cerevisiae* Strain

The yeast strain deleted for the *UBC4* and *UBC5* genes, (*ubc4/5Δ*), MHY508 (*MATa ubc4-Δ1::HIS3 ubc5-Δ1::LEU2 his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1*) was used for the library screen and phenotype analysis. This strain was a gift from M. Hochstrasser.

### Transformation of library plasmids into yeast

The library screen required that two plasmids be cotransformed into the yeast strain MHY508, which is deleted for the *UBC4* and *UBC5* genes and is extremely inefficient for transformation regardless of the procedure used. The *URA3* based library plasmids were introduced with the *TRP1* based RAD6/UBC4 chimeric plasmid using the PLATE transformation procedure (Elbe, 1992). For each transformation, 10 µl of herring sperm DNA (boiled and rapidly cooled before use) was mixed with the yeast cells, followed by the addition of 5 µl of plasmid DNA(s). Library plasmid DNA was prepared as sequencing grade DNA from *E. coli* stocks carrying the library inserts. Likewise, the RAD6/UBC4 chimaera plasmid DNA was also a highly concentrated sequencing grade DNA stock. The cells were then plated on selective SD plates to select for the plasmid(s). Dropout media lacking uracil and tryptophan was used to select for the cotransformation of both the RAD6/UBC4 chimeric plasmid and a library plasmid.

### Isolation of library plasmid DNA from Bacteria

Plasmid DNA for use in restriction enzyme analysis and subcloning was prepared using a modification of the alkaline lysis mini-prep procedure (Birnboim and Doly, 1979) as outlined in detail in Appendix A. The isolation of plasmid DNA from the cotransformed *ubc4/5Δ* yeast strain resulted in a mixture of *URA3* and *TRP1* plasmids. Both plasmids can be efficiently transformed into *E. coli*, as both can be selected for by the ampicillin resistance marker present. Five *E. coli* colonies were selected from each transformation, and the DNA was isolated from each bacteria culture. The *URA3* library plasmid were distinguished from the *TRP1* plasmids by the relative sizes of the uncut plasmids. The smaller ~8kb *TRP1* plasmids were easily distinguished from the library plasmids due to the ~10 kb yeast genomic DNA insert and the resulting increase in molecular weight in the *URA3* plasmids.

**Enzymatic Manipulations and Analysis of the library plasmids**

The genomic inserts present on the isolated plasmids from heat resistant yeast strains were analyzed by restriction site mapping and Southern analysis. The methods describing restriction digestion of DNA and the separation of the resulting fragments has been previously described (See Appendix A). The YEp24 plasmid (see NEB catalogue) was the parental plasmid for the library and was used routinely as a control vector for restriction analysis, Southern analysis, and phenotype analysis.

Southern analysis was performed using the non radioactive digoxigenin (DIG) DNA-labeling and Detection Kit from Boehringer Mannheim. Approximately 2 ng of DNA was used to create each probe. This DNA was purified from LMP agarose gel and labeled according to the DIG labeling protocols provided. The plasmid DNA to be analyzed was digested with appropriate restriction enzymes, and electrophoretically separated on a 1% agarose gel (1% agarose (w/v), 2 mM EDTA, 40 mM Tris-Acetate pH 8.5) in TAE buffer according to Ausubel *et al* (1990). Gels were strained with ethidium bromide and photographed under UV light. DNA was transferred to Hybond-N membrane by capillary blotting in 20 x SSC and immobilized to the membrane by UV crosslinking. The DNA was subject to hybridization with denatured DIG-labeled DNA probe using the pre-hybridization and hybridization steps recommended for this system. Hybridization was carried out at 60°C (12 hours) and blots were washed and processed as the instructions suggested.

Detection of the hybridized probe was done using the DIG specific antibody, anti-digoxigenin-AP conjugate (PPD, Boehringer Mannheim) according to the protocols provided. After incubation with the developer solution, the nylon membrane with the bound probe was exposed to X-ray film and processed to visualize the bands.

**Preparation of DNA for sequencing**

The preparation of sequencing grade DNA is described elsewhere (Appendix A). To sequence the DNA inserts present at the unique *BamH*I site in the YEp24-based *URA3* high copy library, or for sequences subcloned into the *BamH*I site of the *URA3* based YEp352 vector, two primers complementary to either side of the *BamH*I restriction site, located in the tetracycline resistance marker of either, were used. Sequencing was done by the DNA Sequencing and Synthesis Facility in the Department of Biochemistry, at the University of Alberta.

**Amplification of the *URA3* and *REP1* genes by the Polymerase Chain Reaction (PCR).**

Oligonucleotides, synthesized by the DNA Synthesis Facility in the Department of Biochemistry at the University of Alberta, were used as primers in PCR reactions to amplify the *URA3* and *REP1* gene sequences. The general PCR method is described elsewhere (Appendix A). The *REP1* primers are as follows:

5' primer (REP1-N) GCTAGAATTCGAGCTCATGAATGGCGAGACTGC. The *Sst*I restriction site is underlined.

3' primer (REP1-C) GCTAGGTACC[TCA]CCCATCCACCTTTCGCTC. The KpnI restriction site is underlined, and the STOP codon is bracketed. The URA3 primers are as follows:

5' primer (URA3-N) GCTAGAATTCGAGCTCATGTCGAAAGCTACATATAAGG. The *Sst*I restriction site is underlined.

3' primer (URA3-C) GCTAGGTACC[TCA]GTTTTGCTGGCCGCATCTTC. The *Kpn*I restriction site is underlined and the STOP codon is bracketed.

For both PCR reactions, an isolated library plasmid, R3.2, containing the intact *REP1* and *URA3* genes was used as template DNA. The sequence of the *REP1* gene was verified by double stranded DNA sequencing. The *URA3* gene was deemed functional based on its ability to complement a URA3 defect in the *ubc4/5Δ* strain.

**Construction of *REP1* and *URA3* expression vectors**

The *REP1* and *URA3* genes amplified by PCR were placed on a *URA3*-based high copy yeast expression vector behind the *CUP1* promoter. A representation of the relevant portions of this expression vector has been previously described in Figure 2.1. The REP1 and URA3 PCR products replaced an unrelated gene residing within the *Kpn*I/*Sst*I restriction sites. The *URA3* PCR product was also introduced onto an identical vector with the *TRP1* marker replacing the *URA3* marker.

The RAD6/UBC4 chimaera, a plasmid lacking any genes, the wild type *UBC4* gene and wild type *RAD6* gene were likewise expressed from an identical yeast vector, except that the *URA3* plasmid selection marker was replaced with the *TRP1* delectable marker.

**Nucleotide and Protein Sequence Database Searching**

Deduced nucleotide sequences and predicted protein sequences obtained by sequencing and sequence translation were compared to the nucleotide sequence databases (Genbank, EMBL) and peptide sequence databases (SWISS-PROT) at the National Center

for Biotechnological Information (NCBI) using the BLAST algorithm (Altschul *et al*, 1990).

**Subcloning of the library plasmids**

The 2 μ sequences integrated into the YEp24 *URA3* vector (R3.2) contained the open reading frames for all known 2 μ–encoded genes, including *FLP*, *RAF*, *REP1* and *REP2*.

a. Cloning of the 1.3 kb *Hind*III fragment.

The 1.3 kb DNA fragment identified by Southern analysis as being common to all isolated library plasmids was introduced into the single *Hind*III restriction site of the *URA3*-based high copy yeast plasmid YEp352. YEp352 differs from YEp24 parental only in the presence of a multiple cloning site (MCS) in the *LacZ* gene, although the MCS is at the corresponding position of the *BamH*I site in YEp24. The basic cloning techniques outlined in Chapter 2 were used. Sequencing of this insert was accomplished using universal forward and reverse primers available from the Sequencing facility.

b. Cloning of the 3.2 kb *Xba*I fragment.

The inverted repeats of the 2 μ circle contain unique *Xba*I restriction sites which were used to partition the 2 μ sequences into two halves. Within the 3.2 kb *Xba*I-*Xba*I fragment were the sequences encoding for RAF and REP1 proteins as well as the noncoding centromere (CEN)-like sequence and the 2 μ replication origin. A library plasmid containing the entire 2 μ sequence was digested with *Xba*I and the 3.2 kb fragment was ligated into the unique *Xba*I site present in YEp352, described above.

**Relative plasmid copy number determination**

To determine the relative *in vivo* abundance of the library plasmids (containing the complete 2 μ sequences) and the *TRP1*-based *ts* plasmid, total plasmid DNA isolated from the *ubc4/5Δ* yeast strains, composed of a mixture of the *TRP1* and *URA3* plasmids, was subject to Southern analysis using two consecutive probes, the first against the *RAD6/UBC4* gene of the *TRP1* plasmid, and the second against the *URA3* marker. Scanning densitometry of the resulting X-ray films was used to determine the ratio of *URA3* plasmid relative to the *TRP1* based plasmids.

Equal number of cells ($1.29 \times 10^8$ cells) from cotransformed *ubc4/5Δ* yeast strains were used for plasmid DNA isolations. Cell numbers were determined using a Coulter counter, as previously described. Each strain was transformed with the RAD6/UBC4 *TRP1* plasmid, and one of the following *URA3*-based plasmids; complete 2 μ circle (R3.2), YEp24 parental control, or wild type UBC4. The final DNA pellet for each was

resuspended in 100 µl of milliQ water, followed by four 1:10 dilutions (90 µl DNA into 10 µl milliQ water) to provide a range of DNA concentrations.

A control DNA sample was a plasmid miniprep of DNA carrying both the *URA3* marker and the RAD6/UBC4 gene, such that the probes specific for either sequence would detect this DNA. The abundance detected by both plasmid was compared, normalized, and the conversion value was applied to the plasmid mixtures obtained from the different yeast strains. Control DNA was quantitated using a fluorometer, and serially diluted into 100 µl such that four DNA samples containing 1.0 ng, 100 pg, 10 pg and 1.0 pg total DNA were available as controls. For all 100 µl DNA samples, 40 µl of each was diluted into 60 µl of 1x SSC. 3 µl 10 N NaOH was mixed in and the samples heated to 70°C for 45 minutes to denature the DNA. The tubes were then rapidly cooled on ice, and 15 µl of 7.5M ammonium acetate was mixed in and the resulting mixtures were then kept on ice.

Southern analysis was done after the four dilutions of each DNA sample were individually applied to Hybond-N membrane using a Slot Blot apparatus. The entire sample of each was loaded into the wells and allowed to sit for 15 minutes at room temperature. Light suction was applied to slowly draw samples through to the Hybond-N nylon membrane. Next, the wells were washed with 200 µl 1x SSC and this was drawn through by light suction. The DNA was cross linked to the membrane by exposure to UV light.

The URA3 and RAD6/UBC4 probes were digoxigenin (DIG) labeled, and the prehybridization, hybridization, and band visualization steps were done following recommended protocols. First, the URA3 probe was hybridized and the Southern developed, followed by stripping and re-probing with the RAD6/UBC4 probe. Efficiency of stripping was monitored by the disappearance (after the stripping and re-probing procedures) of a band containing URA3-DNA only, spotted onto the membrane.

The relative intensity of the signal for the URA3 probe and the RAD6/UBC4 probe, for the same DNA dilutions, was assumed to represent the relative abundance of the *TRP1* and *URA3* plasmids *in vivo*. Exposed X-ray film was quantitated by densitometry after selecting the DNA concentration with film exposures that fell within the linear range of film density, followed by the densitometric scanning of the appropriate bands, using the Joyce-Loebl Chromoscan 3 scanning densitometer. Integration values for the signals generated by scanning the film represented the relative intensity of each signal, and were assumed to represent the amount of DNA detected by each probe. The ratio of values for the control DNA from both the URA3 and RAD6/UBC4 probe reflected their different specific activities. The difference was calculated, and this correction value was applied to adjust the values of the plasmid mixtures isolated from yeast. The background detection of

the genomic *RAD6* gene, present in the *ubc4/5Δ* strain, by the RAD6/UBC4 probe was assumed to be constant for all samples and was not corrected for.

The relative amount of the *TRP1* and *URA3* plasmids was then reported as the ratio of the integration values obtained by densitometry for one probe to the other for a given DNA mixture and at a given DNA concentration. This ratio is assumed to reflect the ratio of the *TRP1* plasmid to the *URA3*-based library, or a control *URA3* plasmid.

## 5.3 Results

### 5.3.1. A basis for the identification of proteins that interact with a UBC4-like E2.

There were two prerequisites for a genetic screen to identify proteins which associate with UBC4 to modify UBC4 activity. First, the introduction of the library DNA was tested for its ability to confer growth at the nonpermissive temperature. Secondly, such proteins must function only in association with the UBC4 protein, and not function alone by bypassing the requirement of UBC4. A library screen using a conditional mutant, *ts* for UBC4 function, was done using a mutated RAD6 protein which exhibits both complete RAD6 function, and partial UBC4 function (see Chapter 4 for complete phenotype analysis).

The RAD6/UBC4 chimaera and the yeast genomic library were coexpressed from high copy number plasmids and the detection of growth at the nonpermissive temperature was the initial screen toward the identification of interacting protein factors (Figure 5.1). After allowing for growth of the transformants at the permissive temperature, each of the approximately 17,000 colonies were replica-plated and incubated at the nonpermissive temperature of 35.5°C. When a viable colony was observed after six days incubation, the corresponding colony from the 30°C master plate was used for further analysis. Each temperature resistant colony was then streaked out for isolated colonies to avoid cross contamination and to generate a 'pure 'strain of yeast carrying both plasmids. Lastly, two colonies from each 'pure' strain were then re-tested for their ability to grow at the nonpermissive temperature. Using this procedure, 22 individual yeast strains were isolated which were capable of growing at the nonpermissive temperature.

The second condition of the screen was that proteins isolated as associated factors for UBC4 function must suppress the *ubc4/5Δ* defect only in the presence of the *ts* E2. Library plasmids were tested, therefore, for their temperature sensitivity when the RAD6/UBC4 chimaera was absent. If growth was observed at the nonpermissive temperature, then that yeast strain was not retained for further analysis in the screen, as the

growth was not dependent on the presence of the *ts* mutant. Each strain was also tested for the ability to grow at the nonpermissive temperature when the *URA3* library plasmid was lost, but the RAD6/UBC4 chimaera remained, to ensure that heat resistance was not due to an anomalous mutation of the strains themselves. Yeast strains able to grow at the nonpermissive temperature in a RAD6/UBC4 dependent manner were retained for further analysis.

A second round of screening was begun (Figure 5.1). The library plasmids were isolated from each of the yeast strains, passed though *E. coli* for amplification, and tested for their ability to confer growth at the nonpermissive temperature when reintroduced into the *ubc4/5Δ* yeast strain with the RAD6/UBC4 chimaera. The 10 resulting library plasmids that met the conditions of this second screen were then analyzed to identify the gene or genes encoding proteins capable of conferring RAD6/UBC4 dependent growth at the nonpermissive temperature.

*Analysis of isolated library DNA.*

Independent library plasmids capable of conferring growth at the nonpermissive temperature were examined by Southern and restriction enzyme analysis. The YEp24 library parental plasmid was used as a probe in Southern analysis to identify nonhybridizing DNA restriction enzyme fragments, as such DNA sequences would be unique to the genomic DNA insert. However, it was observed that few unique DNA sequences were released by the restriction digests, and furthermore, that the size of the unique DNA could not be rationalized in terms of the size of the isolated plasmids, as the smallest isolated library plasmids appeared to have at least 6 kb of additional DNA (data not shown). Restriction analysis was then carried out to determine the size of the DNA inserted at the *BamH*I site of the various isolated library plasmids. The restriction sites were chosen which flanked the *BamH*I insert site, to facilitate the release of the predicted ~10 kb genomic inserts from the YEp24 parental plasmid. Surprisingly, it was found that some isolated plasmids did not contain an insert (data not shown). Nevertheless, every plasmid appeared to have at least 6 kb of additional DNA at an unknown position on the plasmid and was capable of complementing for the growth defect at the nonpermissive temperature.

Restriction enzyme mapping of the smallest library plasmid, R3.2, was carried out to attempt to find the position, and determine the identity, of the additional DNA. However, the mapping was complicated due to the apparent duplication of DNA sequences within the plasmid (data not shown). Moreover, similar duplications were found in other selected library plasmids. Additional Southern analysis was done to attempt to identify

even small portions of the library plasmids which were unique from the parental vector, using the YEp24 probe. Of eleven different restriction digests tested, one successfully released a DNA fragment which was not contained in the YEp24 parental plasmid.

This 1.3 kilobase (kb) fragment was used as a probe in Southern analysis to test for its presence in the other isolated library plasmids. As shown in Figure 5.2a, a *Hind*III restriction digest of a variety of library plasmids, including R3.2, released a similar 1.3 kb band that was not present in the YEp24 parental. Southern analysis (shown in Figure 5.2b) with the 1.3 kb probe revealed that the 1.3 kb fragment was present in all library plasmids tested. Furthermore, this DNA sequence was unique to the isolated plasmids, as the probe did not hybridize with the parental YEp24 plasmid (Lane C).

### 5.3.2. The presence of an integrated copy of the yeast 2μ circle allows for growth of the temperature sensitive strain at the nonpermissive temperature.

The unique fragment of DNA present in the isolated library plasmids was then subcloned into a plasmid with an appropriate polylinker and sequenced (see Materials and Methods). A database search for homologous DNA sequences in yeast revealed a perfect match with a region of the yeast 2 micron (2 μ) circle. The yeast 2 μ circle is a plasmid present at approximately 100 copies per cell (Armstrong *et al.*, 1989; Gunge *et al.*, 1983). Encoded on this 6.3 kDa plasmid are four known protein products and an origin of replication. A linear map of the 2 μ circle is shown in Figure 5.3, indicating the 1.3 kb *Hind*III fragment identified by the library screen. It was then of interest to determine how much additional 2 μ sequence was present in the isolated library plasmids.

Restriction digests were designed which would release a DNA fragment of predictable size from particular regions of the 2 μ circle. The presence of the *REP1*, *REP2*, and *RAF* genes, encoding 3 of the 4 known 2 μ proteins was determined, as were the sequences spanning the region between the two inverted repeats. Library plasmids, all able to confer heat resistant growth, were digested with the appropriate restriction enzymes and analyzed. In contrast to the control plasmid YEp24, all of the tested plasmids contain at least REP1, REP2, RAF, and probably the entire 2 μ circle (Figure 5.4).

The presence of the 2 μ circle on the library plasmids at a position other than the *BamH*I library insert site was unexpected. An examination of the original, unselected library DNA by restriction analysis indicated that this occurrence was a rare phenomenon. Therefore, the presence of complete 2 μ sequences in the successfully isolated library plasmids indicated that these sequences had been specifically selected for and were not due to their over-representation in the original library DNA.

As previously noted, high copy yeast plasmids routinely carry 2 μ sequences which include the 3' end of the *FLP* gene and the 5' end of the *RAF* gene, and the STB locus (centromere-like stability locus; Storms *et al.*, 1979). Furthermore, the YEp24 parental plasmid does not complement for *ts* growth (Figure 4.3), therefore the additional 2 μ sequences present in selected library plasmids specifically suppress the E2 *ts* defect.

A detailed restriction map of the R3.2 plasmid was then constructed to determine where the 2 μ circle lay within the library plasmid. The resulting restriction map of the R3.2 plasmid, shown in Figure 5.5b, revealed that the entire 2 μ circle had apparently become integrated via the single inverted repeat sequences normally present in the YEp24 vector (see Figure 5.5a) and the two inverted repeats (IR) of the 2 μ plasmid. The resulting plasmid, therefore, contained the four known 2 μ specific proteins, over 2 kb of repeated 2 μ DNA sequences, and a duplication of the replication origin.

### 5.3.3. The integrated 2μ circle results in an increase in plasmid copy number.

Aside from the additional 2 μ sequences present on the R3.2 plasmid, there is another effect resulting from 2 μ integration. The introduction of the complete 2 μ circle onto engineered plasmids has been reported to increase the plasmid copy number to approximately 100 per cell (Ludwig and Bruschi, 1991; Futcher, 1988; Romanos, 1992). This is higher than the approximately 20-40 copies per cell for yeast plasmids carrying the normal *Eco*RI-*Eco*RI fragment of the 2 μ sequence (as in YEp24). Therefore, a higher plasmid copy number may result in higher levels of protein being expressed from the *URA3* library plasmid. To test the possibility that the copy number of the library plasmids was affected by 2 μ integration, the relative copy number of plasmids with, and without, 2 μ DNA sequences was investigated.

If the 2 μ sequences increased the plasmid copy number as suggested by the literature, then the *TRP1*-based RAD6/UBC4 expression vector and the 2 μ containing library plasmids should be present at different levels *in vivo*. Furthermore, the YEp24 library parental plasmid, lacking the 2 μ sequences, should be present at comparable levels to the *TRP1* based plasmid, as both contain identical portions of the 2 μ circle. This was tested directly by isolating *TRP1* and *URA3* plasmid mixtures from various doubly transformed *ubc4/5Δ* strains. The ratios of *TRP1* and *URA3* plasmid were determined by Southern analysis using probes specific for either plasmid DNA, followed by quantitation of their respective signals (see Materials and Methods). The RAD6/UBC4 *TRP1* plasmid and the YEp24 plasmid control isolated from yeast were found to be at near equivalent levels *in vivo* (Table 5.1) Three individual experiments yielded URA3:TRP1 ratios of

(1.6):1, (1.4):1 and (1.5):1. In contrast to this, the ratio between a plasmid containing the entire 2 μ sequence (R3.2 library plasmid) and that of the RAD6/UBC4 plasmid was determined to be at (5.53):1, (4.73):1 and (5.50):1 in three separate experiments (Table 5.1). This result, therefore, suggested that the integration of the 2 μ sequences into the YEp24 plasmid resulted in an increase in copy number of the 2 μ-containing plasmid, as originally reported.

The increase in copy number of the library plasmids may result in the simultaneous increase in expression of proteins from these plasmids. We wanted to determine if the greater copy number of a single gene product was responsible for the temperature complementation. Candidate proteins whose overexpression may confer growth at the nonpermissive temperature include both those encoded by the 2 μ circle, and those encoded by the YEp24 plasmid. However, the 2 μ circle, and therefore the proteins encoded by it, are not essential for normal yeast function , as yeast strains lacking the endogenous 2 μ circle (referred to a cir° strains) exhibit no obvious phenotype (Futcher *et al.*, 1988; Nestmann *et al.*, 1986). For this reason, other plasmid-encoded proteins were initially investigated for their ability to confer temperature resistance when overexpressed.

In addition to the four 2 μ–encoded proteins, the R3.2 plasmid also expressed the *URA3* selectable marker encoding for the orotidine-5'-P-decarboxylase gene (called the *URA3* gene, for simplicity). The *URA3* gene complements the *ura3* mutation in yeast, enabling *ura3* cells to survive without an exogenous supply of uracil by providing a necessary enzymatic step for uracil biosynthesis. In general, yeast cells proliferate at a slower rate in accordance with the number of plasmids present, suggesting that the nutritional needs met by plasmid marker expression are barely sufficient. This reasoning has been used to explain why a yeast strain grown in rich media proliferates at a faster rate than the same stain grown in selective media for plasmid maintenance. Furthermore, when exposed to higher temperatures such as those used in the library screen, this additional heat stress may result in loss of cell viability. It was hypothesized that overproduction of the *URA3* gene would relieve such nutritional stress, which in turn may lower the overall stress of the cell. This putative decrease in cellular stress may have resulted in the observed increased cell viability and proliferation at the nonpermissive temperature.

### 5.3.4. The complementation of the *ts* strain is not due to the increased expression of the *URA3* gene.

It was proposed that the overexpression of the *URA3* selectable marker gene may relieve nutritional stress and thereby result in a greater cell viability at 35.5°C. To test this hypothesis, the *URA3* gene was amplified by PCR (see Materials and Methods) and cloned

onto a high copy yeast plasmid behind the control of the *CUP1* promoter. After transformation of this plasmid with the *TRP1* RAD6/UBC4 chimaeric plasmid, the effect on complementation by *URA3* overexpression was tested. The *CUP1* promoter can increase expression up to 50 fold (Butt *et al.*, 1987), and, therefore, growth in the presence of copper would be expected to mimic the copy number effects due to 2 μ presence.

The *ubc4/5Δ* strain transformed with the RAD6/UBC4 *TRP1* plasmid, and either wild type UBC4, a null marker plasmid, or the *URA3* gene, was tested for the ability to grow at the nonpermissive temperature in the presence of copper. As shown in Figure 5.6a, the three *ubc4/5Δ* strains grow efficiently at 30°C. In contrast to this, yeast strains expressing the RAD6/UBC4 plasmid alone, or with, the *URA3* gene, were both unable to grow at the nonpermissive temperature of 35.5.°C (Figure 5.6b). From this result, it appears as though the overexpression of *URA3* is not sufficient to complement for growth at the nonpermissive temperature. Therefore, this result implied that the increased copy number of the *URA3* gene resulting from the integration of the 2 μ circle was not responsible for the complementation of growth at the nonpermissive temperature.

### 5.3.5. The DNA sequences between the 2μ inverted repeats encode for two proteins and can partially complement for the *ts* phenotype.

The proteins encoded by the 2 μ circle were then investigated for their involvement in conferring temperature resistant growth. As an initial test, a portion of the 2 μ circle was tested for its complementation ability in the absence of the other 2 μ sequences. An internal *Xba*I-*Xba*I fragment of the 2 μ circle, encoding for the *REP1* and *RAF* genes was used (Figure 5.3). This 3.2 kb DNA fragment was tested for its ability to complement for growth at the nonpermissive temperature, shown in Figure 5.7.

The *ubc4/5Δ* strain was transformed with the RAD6/UBC4 plasmid, as well as wild type UBC4, a null *URA3* marker plasmid (YEp24), the R3.2 plasmid containing the entire 2 μ sequences, and the R3.2X plasmid containing the *Xba*I-*Xba*I 2 μ circle fragment. At 30°C, all four strains were capable of growth, although none to the level of wild type (Figure 5.7a). However, at 35.5°C, a distinct effect on growth was observed. As shown in Figure 5.7b, the RAD6/UBC4 plasmid alone was severely deficient in growth at the nonpermissive temperature, even at the lowest cell dilutions. Cells containing the R3.2 plasmid, however, were able to grow at the nonpermissive temperature, as had been previously observed in the library screening process. Significantly, the R3.2X plasmid was also capable of sustaining noticeable growth when coexpressed in the *ubc4/5Δ* strain at the nonpermissive temperature. This result suggested that one, or both, of the two 2 μ encoded genes present on the R3.2X vector was involved in conferring cell viability at the

nonpermissive temperature. However, it was also observed that the R3.2X plasmid had significantly fewer visible colonies as compared to the full 2 μ plasmid (R3.2), suggesting that there was limited complementation for growth at the nonpermissive temperature. This may have been a direct result of the lower copy number of the *REP1* and *RAF* genes expressed from the vectors, as the absence of the complete 2 μ circle was predicted to decrease the plasmid copy number back to 20 to 40 per cell. Therefore, it was possible that the gene products require the elevated copy number levels provided by the complete 2 μ integration in order for full complementation to be observed, as the *Xba*I-*Xba*I fragment is not behind a strong inducible promoter and therefore, the expression levels are not induced.

### 5.3.6. Overexpression of *REP1* affects the growth of the *UBC4/5* deletion strain at permissive and nonpermissive temperatures.

The previous observation that a portion of the 2 μ circle expressing only the *REP1* and *RAF* genes was able to partially reconstitute the growth phenotype suggested that one of these genes was a likely candidate for a protein involved in temperature resistance at the nonpermissive temperature. To directly test the possibility that overexpression of these genes was required for full complementation, we first amplified the *REP1* gene by PCR and placed it behind the inducible copper promoter (see Materials and Methods). The overexpression of *REP1* was then tested for its ability, in association with the RAD6/UBC4 chimaera, to confer growth at the nonpermissive temperature. Because the following experiments yielded positive results, the *RAF* gene was not tested.

The *ubc4/5Δ* strain was transformed with the RAD6/UBC4 plasmid as well as either wild type UBC4, a null *URA3* marker plasmid, the R3.2 plasmid or the *REP1* gene behind the *CUP1* promoter. At 30°C, all four strains were observed to grow efficiently in the presence of copper (Figure 5.8a). It was noted, however, that the *ubc4/5Δ* strain formed larger colonies when coexpressing *REP1* (column IV) as compared to a null plasmid (column II) or the R3.2 plasmid (column III). To determine if this observation reflected an increase in the growth rate, a qualitative assessment of growth rate was performed when the *UBC4/5* deletion strain expressed the *RAD6/UBC4* chimaera in the presence or absence of *REP1* coexpression (Table 5.2a).

At the permissive temperature, the doubling time of the *ubc4/5Δ* strain expressing the *RAD6/UBC4* derivative with a *URA3* null vector was 4.3 hours, yet when the *REP1* gene was coexpressed with the RAD6/UBC4 plasmid, the doubling rate decreased to 3.5 hours, reflecting the increased cellular growth rate. It should be noted that the doubling time for the strain producing the RAD6/UBC4 chimaera alone (Table 4.1, 3.85 hr) was different than that of the RAD6/UBC4 chimaera coexpressed with the URA3 vector (4.3

hr). The slower growth rate of the latter strain was attributed to the amplified nutritional stress induced by having two nutrient requirements dependent on plasmid expression rather than the one previously used. This data indicates that the expression of *REP1* with the *RAD6/UBC4* gene results in an increase in the growth rate of the *UBC4/5* deletion strain.

At the nonpermissive temperature of 35.5°C, the expression of the RAD6/UBC4 plasmid in the *UBC4/5* deletion strain did not promote cellular growth (Figure 5.8, column II), as had been previously observed (Figure 5.6 and 5.7). Moreover, this RAD6/UBC4 chimaera was unable to grow at the nonpermissive temperature despite the predicted 50 fold induction from its *CUP1* promoter due to the presence of copper in the plating media. However, when expressed in combination with either the R3.2 or REP1 plasmids, there was visible cellular growth at the nonpermissive temperature (column III and IV). Moreover, the number and size of the colonies formed by strains expressing either the entire 2 μ plasmid (R3.2) or *REP1* alone were similar. These results indicate that the *REP1* gene product is as efficient as the complete 2 μ circle in complementing for the growth of the *ubc4/5Δ* strain at the nonpermissive temperature when coexpressed with the *RAD6/UBC4* chimaera.

### 5.3.7. *REP1* expression increases the growth rate of the *ubc4/5Δ* strain coexpressing Asp95 UBC4.

The ability of the *REP1* gene to increase the growth rate of the *ubc4/5Δ* strain expressing the *RAD6/UBC4* chimaera indicated that *REP1* expression affected UBC4 growth function at the permissive and nonpermissive temperatures. It had been previously observed that a point mutation in UBC4 was impeded in its ability to grow at wild type rates at the permissive temperature (Asp95 UBC4, Table 4.1a). An investigation was initiated to determine if *REP1* expression also affected the growth rate of the *UBC4/5* deletion strain expressing this UBC4 derivative.

The result of this experiment is shown in Table 5.2b. The overexpression of *REP1* increased the growth rate of the yeast strain at 30°C, shown by the decrease in doubling time from that of the Asp95 UBC4 mutant alone (4.5 hr), to that of the Asp95 UBC4 mutant coexpressed with *REP1* (3.2 hr) in the *UBC4/5* deletion strain. As noted before, the doubling time for the *ubc4/5Δ* strain with two plasmids (Asp95 UBC4 and YEp24) was slowed relative to that of a strain expressing only one plasmid (Asp95 UBC4, 4.15 hr doubling time, Chapter 4). These results demonstrate that *REP1* coexpression with the Asp95 UBC4 derivative in the *UBC4/5* deletion strain increased the growth rate compared to the same strain expressing Asp95 UBC4 alone. Therefore, in addition to suppressing

the growth defect of the RAD6 derivative, *REP1* expression also affects the growth defect of this UBC4 derivative.

## 5.4 Discussion

The purpose of this investigation was to identify proteins which interacted with a Ub conjugating enzyme capable of carrying out both RAD6 and UBC4 functions *in vivo* in *S. cerevisiae*. While there are several established methods in yeast now developed for the direct identification of protein-protein interactions, attempts to apply them to UBC4 were not successful. These approaches included both the two-hybrid system, and co-purification of associated protein factors with GST-tagged UBC4 from whole cell lysates using glutathione-linked beads. Instead, a screen of the yeast genome was carried out which was designed to identify genes whose protein products affect the function of a conditional UBC4 mutant. The result of this screen was the identification of a yeast gene, *REP1*, whose overexpression affected the UBC4 growth function.

An E2 derivative, referred to as a RAD6/UBC4 chimaera due to its ability to carry out both RAD6 and limited UBC4 functions (Chapter 4), was found to be temperature sensitive (*ts*). This *ts* strain was used to screen the yeast genome for genes whose protein products conferred growth at the nonpermissive temperature in a RAD6/UBC4 dependent manner. This screen eliminated those proteins able to act alone and therefore bypass the need for UBC4 activity. Based on these requirements, the *REP1* gene was identified and therefore was thought to be a candidate for expressing a protein which interacted with the RAD6/UBC4 chimaera.

REP1 protein, encoded by the yeast 2 μ circle, functions in association with a second 2 μ protein, REP2, to form a heterologous transcriptional repressor complex (REP, **rep**ressor of transcription, reviewed in Murray, 1987). The yeast 2 μ circle actively maintains a high, but stable, copy number in the cell, even though the plasmid confers no selective advantage to its host (Nestmann *et al.*, 1986). Copy levels are maintained by proteins encoded by the plasmid. The 2 μ DNA encodes a stability system consisting of the plasmid replication origin, a site specific recombinase (FLP), and two *trans*-acting functions, the products of the *REP1* and *REP2* genes.

The stability of plasmid copy number in yeast populations is due in part to the ability of the 2 μ circle to be transmitted efficiently to daughter cells during cell division. This process is attributed to the combined actions of two plasmid encoded proteins, REP1 and REP2. The mechanism of plasmid partitioning has been attributed to the anchoring of 2 μ DNA to the nuclear matrix by the C-terminus of the REP1 protein, through C-terminal sequences exhibiting sequence homology to both nuclear lamins A and C as well myosin

heavy chain (Wu *et al.*, 1987). The stability of the 2 μ plasmid is also due to the presence of a centromere- (*CEN*)-like element, the STB locus.

A second reason for the plasmid stability of the 2 μ circle is its capacity for copy number amplification. Plasmid amplification occurs by a novel mechanism, first proposed by Futcher (1986). The structural organization of the 2 μ circle is integral to its method of maintenance (Figure 5.3b). The plasmid consists of two domains separated by two regions of 599 bp, each of which are precise inverted repeats of one another. The plasmid-encoded FLP protein catalyses site-specific recombination at specific sites near the center of the inverted repeats. The result of this recombination event is inversion of the two unique regions with respect to one another. A single origin of replication lies at the junction between one of the repeats and the large unique region. Under normal conditions, plasmid replication forks proceed in a bi-directional manner away from the single origin to yield two copies of the parental plasmid. Futcher noted that if FLP-mediated recombination occurred between the duplicated and nonduplicated repeat during fork elongation, then the relative duplication of the two forks would be inverted. As a consequence, the forks would proceed around the circular plasmid in the same direction, generating numerous copies of the 2 μ plasmid, and would terminate only after a second recombination event. Therefore, the normal replicate mode, theta, was converted to what was referred to as 'rolling circle' replication.

Central to regulation of copy number, therefore, is regulation of FLP-mediated activity. This regulation has been shown to be due to the concerted action of the REP1 and REP2 proteins, which associate into a heterologous transcription repressor complex and act at the *FLP1* promoter (Murray, 1987). Additionally, this complex acts at the *REP1* promoter. This latter ability, therefore, acts as a feedback loop for FLP repression. According to the model, if REP1 protein levels fall significantly due to a decrease in plasmid copy number, then repression by REP1 is lifted, a round of rolling circle replication is initiated, and plasmid copy numbers are again raised (Som *et al.*, 1988). In contrast, overexpression of *REP1* is predicted to severely decrease the copy number of the endogenous 2 μ circle (Murray, 1987), as the REP1-dependent repression of *FLP* would not be alleviated as long as *REP1* is overexpressed from *CUP1*, and a decrease in 2 μ plasmid numbers would not be rectified. Because the *ubc4/5Δ* strain contains endogenous 2 μ plasmids (Seufert and Jentsch, 1990), we were unable to directly test the effect of *REP1* expression on cell viability in the absence of the REP2 protein, as it may be that they function only as the heterodimer. Future experiments should be done to cure the *ubc4/5Δ* strain of the endogenous 2 μ plasmid (Harford *et al.*, 1987), and to test the ability of REP1 to function in the complete absence of the FLP or REP2 proteins.

Despite the identification of REP1 as a protein candidate, this investigation has not directly tested the physical interaction between the REP1 protein and the RAD6/UBC4 protein or how such an interaction plays a role in UBC4 function. This is an obvious direction for further investigations. In contrast to the idea of a UBC4-REP1 interaction, it is also possible that REP1 functions indirectly to lower the levels of a UBC4 growth related target. As discussed above, REP1 levels are inversely correlated with 2 μ plasmid copy number. Therefore, it has been proposed (although not tested) that REP1 overexpression will lead to the decrease of the 2 μ circle (Murray, 1987) and in the decrease of 2 μ encoded protein products such as REP2, RAF and FLP. In view of the apparently benign effects of the 2 μ circle on cellular growth or viability, it is extremely difficult to imagine how this can be related to the UBC4 suppression effect observed here.

The possibility therefore remains that REP1 and UBC4 physically interact. The observation that the REP1 protein only complements for *ts* growth in the *ubc4/5Δ* strain in the presence of either RAD6/UBC4 or Asp95 UBC4 is at least consistent with this idea. Significantly, the amino acid residues at positions 82 (in the RAD6/UBC4 derivative) and 95 (in the Asp95 UBC4 derivative) are physically adjacent on the UBC4 protein (Figure 4.2). It may be, therefore, that the REP1 protein interacts with this face of the UBC4 protein. Nevertheless, proof of a protein-protein interaction between REP1 and UBC4 awaits further investigation.

Several possibilities can be envisioned for the function of REP1 in the UBC4 pathway through physical interactions. One possibility is that REP1 recruits UBC4 to the nuclear matrix where a specific protein component may be targeted for degradation. Alternatively, the observation that REP1 binds to DNA and functions as a transcriptional regulator within a complex presents the possibility that UBC4 functions by targeting one of the associated protein factors. The association among proteins incorporated into transcriptional complexes with Ub conjugating enzymes is well documented. For example, both the MATα2 and GCN4 transcriptional regulator proteins are targeted by Ub conjugating enzymes for degradation (Chen *et al.*, 1993; Kornitzer *et al.*, 1994). Preliminary evidence, moreover, has indicated that the E2F and E2A mammalian transcription factors are also apparently targeted and degraded in a Ub dependent manner (Magae and Loveys, unpublished results). Therefore, it may be that REP1 serves to recruit UBC4 to the DNA to target other proteins for ubiquitination. Yet, the lack of a phenotype associated with loss of endogenous 2 μ circles implies that this potential function of UBC4 with REP1 is not essential, and therefore the result of the possible REP1 and UBC4 interaction cannot easily be determined.

Many questions remain regarding the significance of this study. Certainly, because the 2 μ plasmid is restricted to *S. cerevisiae* and related yeasts, the potential interactions between REP1 and the RAD6/UBC4 protein cannot be applied to higher eukaryotes. It may be, in a manner analogous to the RAD6/RAD18 associations (Bailly *et al.*, 1994) resulting in the predicted recruitment of RAD6 to site of DNA damage, that REP1 acts as a *trans*-acting protein factor to recruit UBC4 to the nuclear matrix where UBC4 carries out an unknown function.

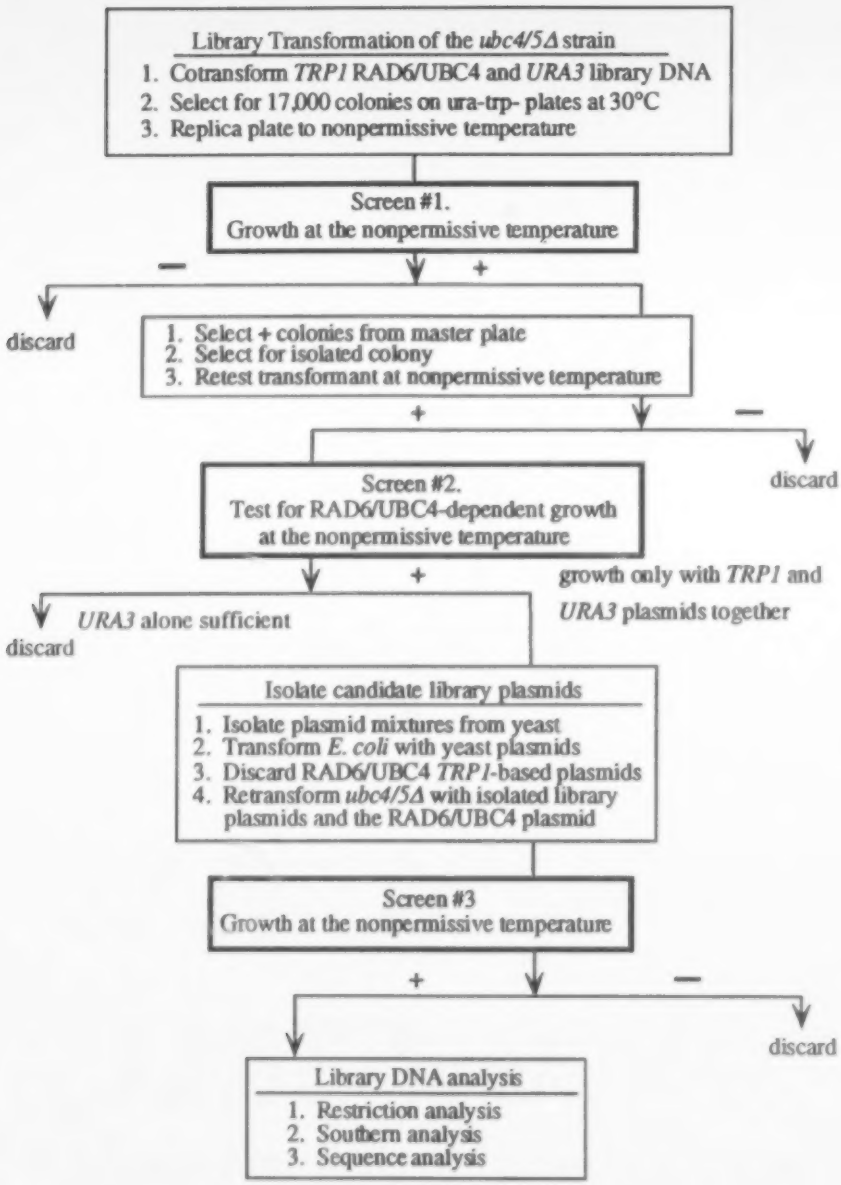



FIGURE 5.1

**Flow chart outlining the order of steps taken in the library screen of *S. cerevisiae* genomic sequences for those protein products which effect UBC4 function at nonpermissive temperature.**

Shown is the outline of the library screen done for the purpose of identifying UBC4-interacting proteins. A full description of the methods referred to are given in the text. - and + refer to the absence or presence, respectively, of detectable growth on SD plates after 6 days incubation at the nonpermissive temperature of 35.5°C.

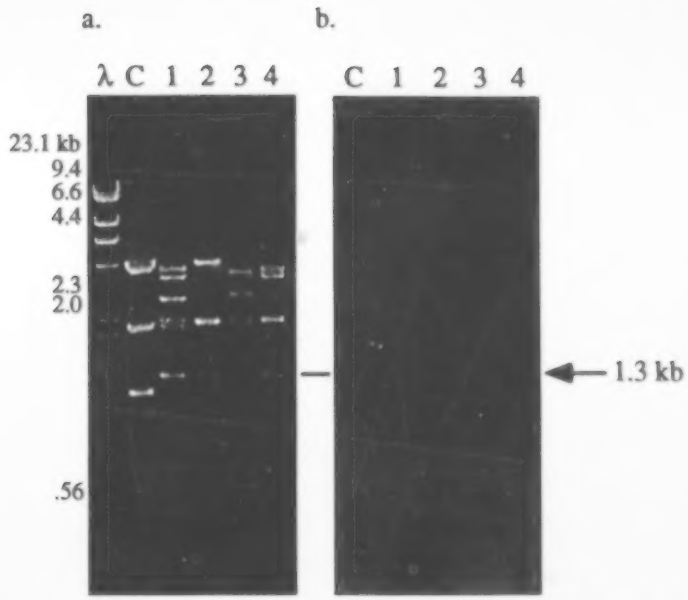



FIGURE 5.2

**A 1.3 kilobase (kb) *Hind*III fragment is common to all library isolates.**

Part a. A *Hind*III restriction digest of four isolated library plasmids (Lanes 1, 2, 3 and 4) and the library parental lacking an insert (YEp24, C lane) reveal a common 1.3 kb DNA fragment unique to the four library plasmids. Lane 2 is the R3.2 plasmid, representing the smallest library plasmid class. The three additional library plasmids (Lane 1, 3, and 4) were of higher molecular weight. The control lane (C) is the YEp24 library a parental plasmid lacking insert DNA.

Part b. Southern analysis of the *Hind*III digested DNA shown in Part a, using the DIG-labeled 1.3 kb *Hind*III fragment noted above as a probe. The 1.3 kb fragment of interest is indicated by an arrow. λ, Lambda *Hind*III DNA standards of the sizes indicated.

FIGURE 5.3

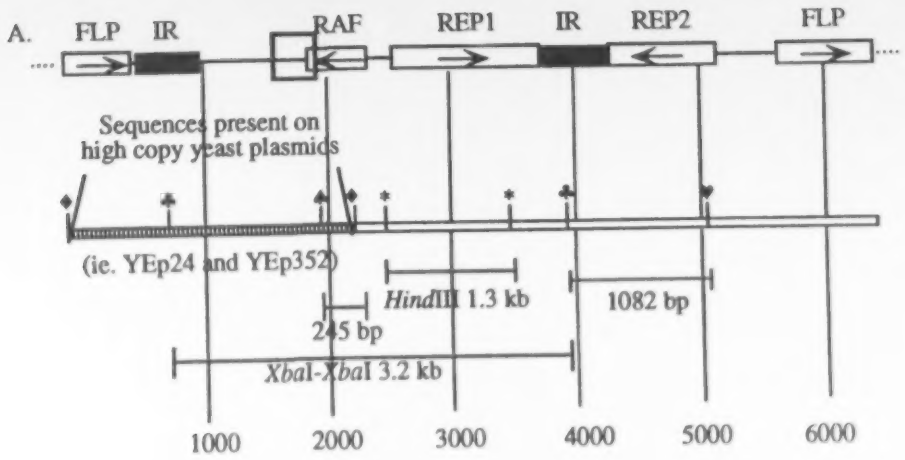
**A linear and circular map of the *S. cerevisiae* β-form 2 μ circle.**

The linear map of the 2 μ circle is shown in Part A. The map is drawn to scale, and features the four open reading frames (ORF) encoding the four 2 μ proteins. The position and identity of the genes is denoted by the named boxes, and the direction of the ORF is shown by the arrows within each. Also indicated is the position and direction of the two inverted repeat sequences, containing 599 bp of duplicated DNA. The noncoding centromere-like STB (**stab**ility) locus is also indicated as a stippled box without an arrow, and overlapping the RAF ORF. The 1.3 kb *Hind*III fragment used in the Southern analysis (Figure 5.2) is indicated

The positions of key restriction enzyme sites are shown (see separate figure legend). The expected size of the 2 μ DNA fragments generated by the restriction enzyme mapping (shown in Figure 5.4) are indicated. Furthermore, the 2.24 kb portion of the 2 μ circle present in standard yeast high copy vectors, such as YEp24 and YEp352, is marked by the hatched rectangle below the linear 2 μ map.

The circular map of the 2 μ circle is shown in Part B and is drawn such that the long inverted repeats are aligned. The STB and origin of replication (ori) are shown. The inverted repeats containing FLP recognition and crossover sites are marked as stippled boxes. (Figure adapted from Murray, 1987).

A.




| Restriction Enzyme | Position (bp) | Symbol legend |
|---|---|---|
| *Nco*I | 5024 | ♥ |
| *Eco*RI | 1, 2238 | ♦ |
| *Pst*I | 1993 | ♠ |
| *Hind*III | 2314, 3628 | * |
| *Xba*I | 703, 3945 | ♣ |

B.

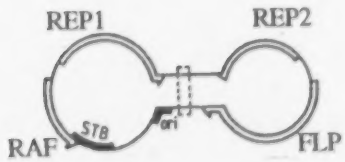


FIGURE 5.4

**DNA Restriction analysis of isolated library plasmids reveals the presence of unique 2 μ DNA sequences.**

Shown are photographs of ethidium bromide stained agarose gels of four different restriction digests (a, b, c, and d) of various *URA3* library plasmid isolated by the genetic screen outlined in detail in the text. The library plasmids are numbered 1 through 10. Number 1 is the R3.2 plasmid, the smallest library plasmid isolated. The library parental plasmid, lacking an insert (YEp24) is the C lane in all four digests. 123 refers to the commercially available 123 bp ladder, and λ refers to lambda *Hind*III size standards, both of which are used for size reference. In each case, the asterix (*) marks the position of the DNA fragment predicted to be released if the unique 2 μ sequences are present.

Panel A. A *Hind*III restriction digest predicted to release a 1.3 kb fragment corresponding to the 2 μ encoded *REP1* gene. Note that the DNA fragments generated after enzymatic digest from library plasmids #1 and #7 likely resulted from partial digestion.

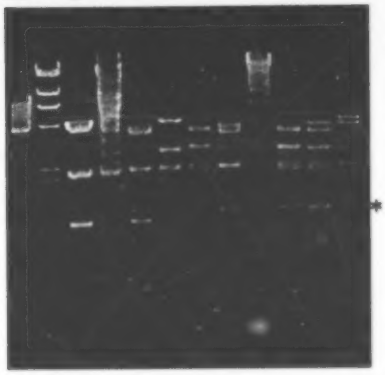
Panel B. An *Xba*I-*Nco*I digest predicted to release a 1,082 bp fragment corresponding to the *REP2* gene encoded by the 2 μ circle.

Panel C. An *Xba*I digest which will release a 3.2 kb fragment correspoding to the inverted repeats (IR), the 2 μ origin of relication, the *RAF* and *REP1* genes, and the STB locus.

Panel D. A *Pst*I-*Eco*RI digest designed to release a 245 bp fragment as a portion of the *RAF* gene encoded by the 2 μ circle. Details of restriction sites and gene positions of the 2 μ circle are given in detail in Figure 5.3, which include the restriction digests referred to in this experiment. Panels A and B were electrophoresed through the same 1% agarose gel, and the molecular weight markers of Panel A therefore apply to Panel B. Panel C shows the digested DNA after electrophoresis through a 0.75% agarose gel to separate the 3.2 kb DNA fragment from nearby DNA fragments. Panel D used a 1% agarose gel.

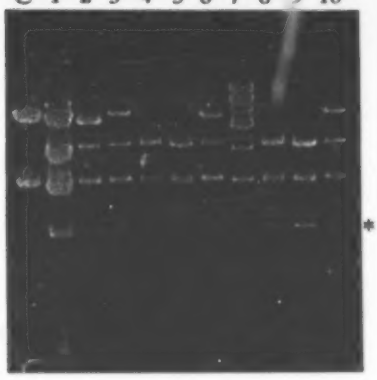
A. 1.3 kb *REP1* fragment

123 λ C 1 2 3 4 5 7 8 9 10



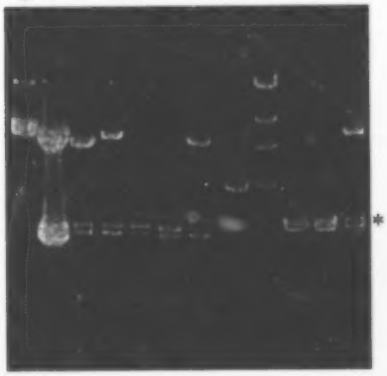
B. 1,082 bp *REP2* fragment

C 1 2 3 4 5 6 7 8 9 10



C. 3.2 kb IR fragment

C 1 2 3 4 5 7 123 λ 8 9 10



D. 245 bp *RAF1* fragment

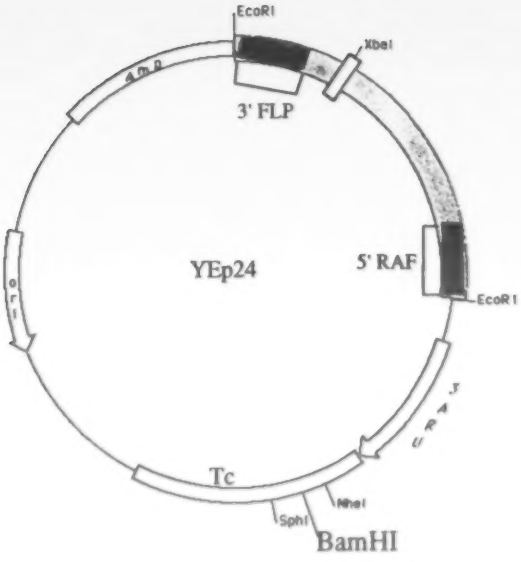
123 λ C 1 2 3 4 5 6 7 8 9 10

FIGURE 5.5

**Comparative plasmid maps of the YEp24 library parental plasmid and the R3.2 library plasmid.**

Two circular plasmid maps of the YEp24 parental (NEB, 1994) and the YEp24 parental with the entire 6.3 kb 2 μ plasmid integrated (R3.2) are shown. Both maps are drawn to scale, and the plasmid organization of each is indicated. Abbreviations are as follows: Amp, ampicillin resistance. ori, origin of replication. Tc, tetracycline resistance. *URA3*, orotidine 5'-P-decarboxylase gene. 2 micron, ori STB: 2.24 kb portion of the 2 μ circle used to create high copy yeast plasmids containing the 2 μ centromere stability (STB) locus.

Also indicated are the relevant restriction enzyme sites. The unique *BamH*I site in the Tc gene was the predicted location for insert DNA. *Xba*I restriction sites are unique to the 599 bp inverted repeat region of the 2 μ circle. The 2.24 kb *EcoR*I fragment present in YEp24 is specifically duplicated in the R3.2 plasmid and is represented by the lightly filled portions of both plasmids. Dark filled rectangles in both plasmid maps represent the location of the ORFs encoding for the FLP, REP1, REP2, and RAF proteins.


EcoRI
Xbal
Amp
3' FLP
YEp24
5' RAF
EcoRI
ori
URA 3
Tc
SphI
NheI
BamHI

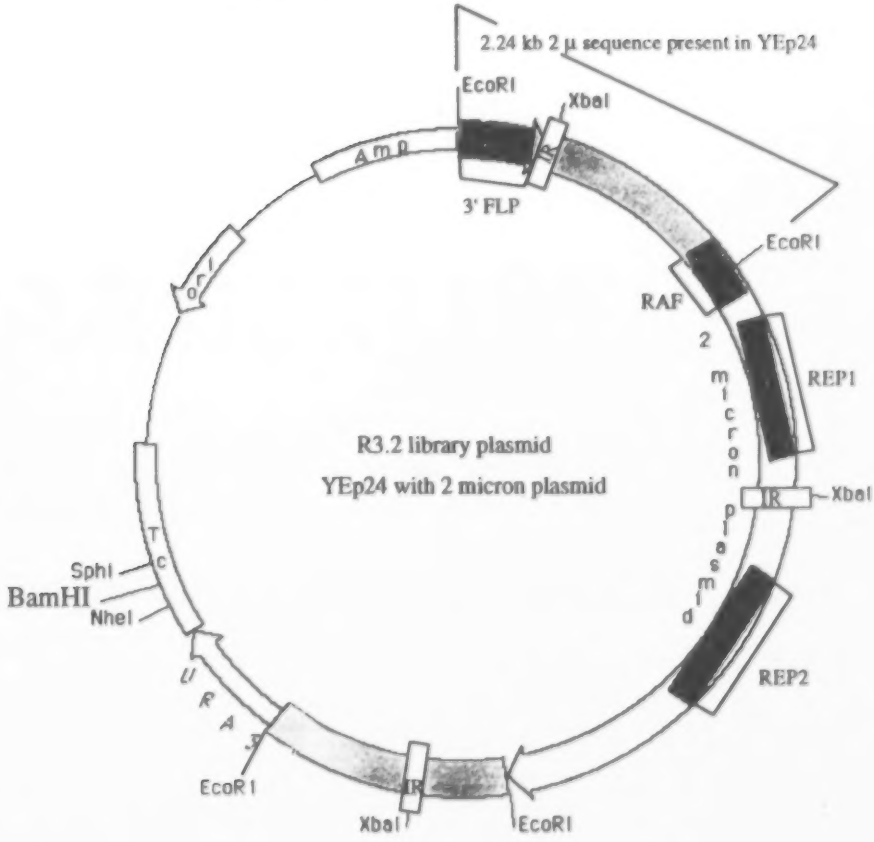
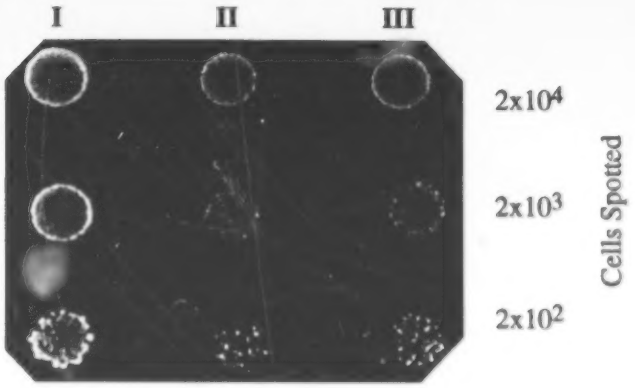

2.24 kb 2 μ sequence present in YEp24
EcoRI
Xbal
Amp
3' FLP
EcoRI
RAF
2
micron
plasmid
REP1
IR
Xbal
ori
R3.2 library plasmid
YEp24 with 2 micron plasmid
Tc
SphI
BamHI
NheI
URA 3
REP2
EcoRI
IR
Xbal
EcoRI

FIGURE 5.6

**The overproduction of URA3 does not complement for growth of the *ubc4/5Δ* strain coexpressing the RAD6/UBC4 chimaera at nonpermissive temperatures.**

The yeast *UBC4/5* deletion strain was transformed with a series of *URA3* and *TRP1* high copy plasmids before testing for growth at the nonpermissive temperature. A mid-log phase culture of each transformed yeast strain was diluted to $2x10^4$, $2x10^3$ and $2x10^2$ cells per 30 µl, spotted onto plates and dried. The plates contained SD medium with copper for *CUP1* promoter induction, and enriched with all amino acids but tryptophan and uracil, for plasmid selection. Cell numbers were determined using a Coulter counter (see Methods). The plates were incubated at 30°C for 4 days (Part a) or at 35.5°C (Part b) for 6 days. The strains were plated in columns at increasing dilutions. **Column I**, wild type UBC4 (*TRP1*) in combination with a *URA3* null plasmid (YEp24). **Column II**, the RAD6/UBC4 temperature sensitive plasmid (*TRP1*) in combination with a *URA3* null plasmid (YEp24); **Column III**, the RAD6/UBC4 plasmid in combination with the URA3 plasmid behind the *CUP1* promoter.

a. 30°C


Yeast Strain
I
II
III
2x10^4
2x10^3
2x10^2
Cells Spotted

b. 35.5°C

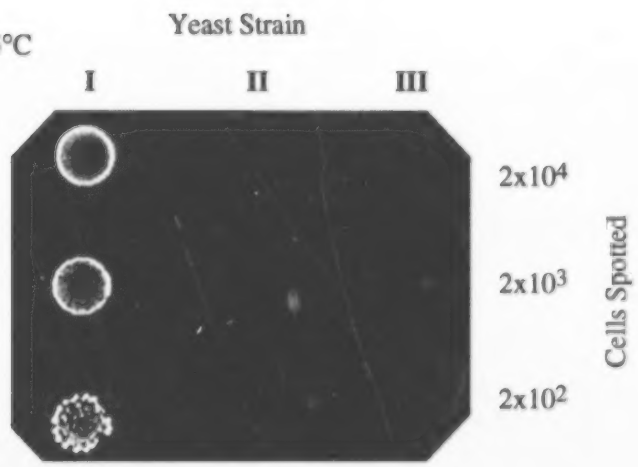
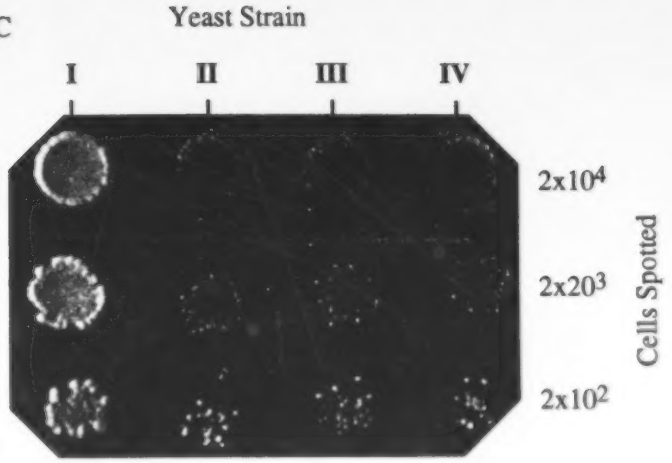

Yeast Strain
I
II
III
2x10^4
2x10^3
2x10^2
Cells Spotted

FIGURE 5.7

**An 3.2 kb portion of the 2 μ plasmid partially complements for growth of the *ubc4/5Δ* strain coexpressing the RAD6/UBC4 chimaera at nonpermissive temperatures.**

The yeast *UBC4/5* deletion strain was transformed with a series of *URA3* and *TRP1* high copy plasmids before testing for growth at the nonpermissive temperature. A mid-log phase culture of each transformed yeast strain was diluted to $2x10^4$, $2x10^3$ and $2x10^2$ cells per 30 μl, spotted onto plates, and dried. The plates contained SD medium enriched with all amino acids but tryptophan and uracil, for plasmid selection. The plates did not contain copper. Cell numbers were determined using a Coulter counter (see Methods). The plates were incubated at 30°C for 4 days (Part a) or 35.5°C (Part b) for 6 days. The strains were spotted in columns and include: **Column I**, wild type UBC4 (*TRP1*) in combination with a *URA3* null plasmid (YEp24). **Column II**, the RAD6/UBC4 plasmid (*TRP1*) in combination with a *URA3* null plasmid (YEp24). **Column III**, the RAD6/UBC4 plasmid in combination with the YEp352 plasmid (see Methods) containing a 3.2 kb *Xba*I-*Xba*I fragment of the 2 μ plasmid (R3.2X). **Column IV**, the RAD6/UBC4 plasmid in combination with the YEp24 plasmid containing the entire 2 μ sequences (R3.2).

a. 30°C


Yeast Strain
I
II
III
IV
2x10^4
2x20^3
2x10^2
Cells Spotted

b. 35.5°C

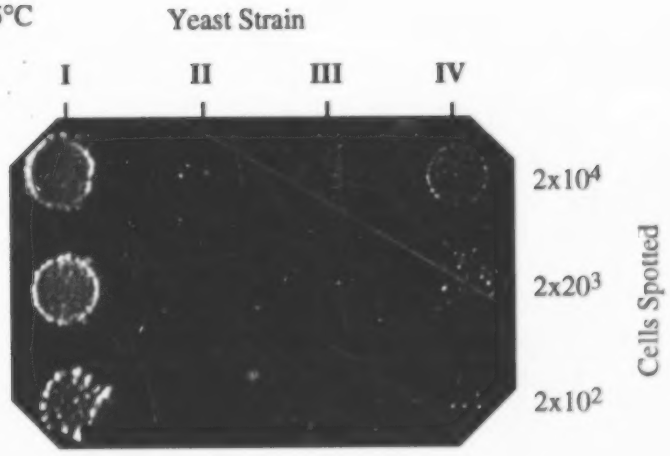
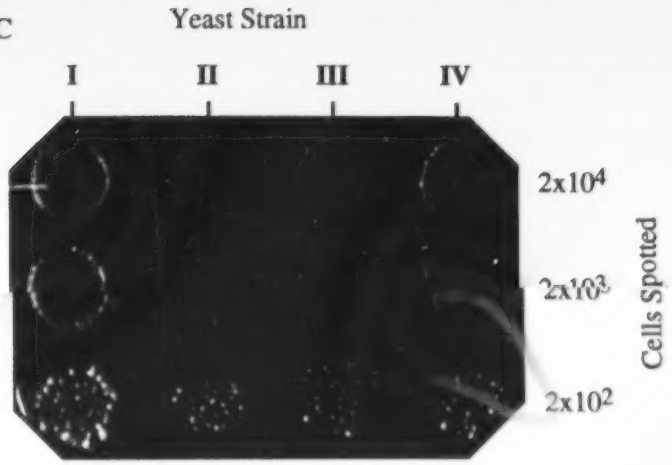

Yeast Strain
I
II
III
IV
2x10^4
2x20^3
2x10^2
Cells Spotted

FIGURE 5.8

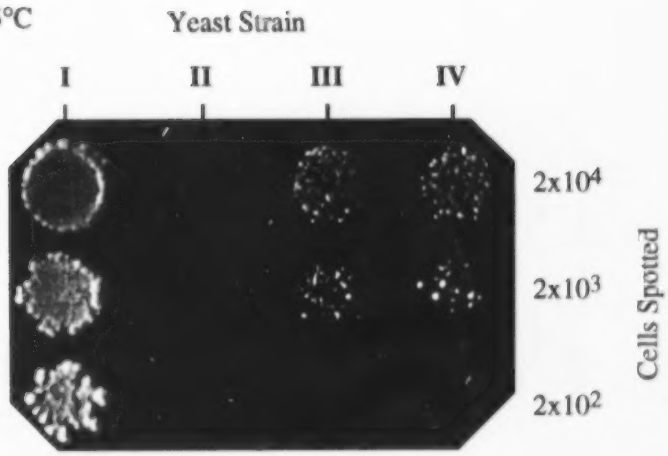
**Overexpression of the 2 μ gene, *REP1*, complements for growth of the *ubc4/5Δ* strain coexpressing the RAD6/UBC4 chimaera at nonpermissive temperature.**

The yeast *UBC4/5* deletion strain was transformed with a series of *URA3* and *TRP1* high copy plasmids before testing for growth at the nonpermissive temperature. A mid-log phase culture of each transformed yeast strain was diluted to $2x10^4$, $2x10^3$ and $2x10^2$ cells per 30 μl, spotted onto plates and dried. The plates contained SD medium with copper for *CUP1* promoter induction, and enriched with all amino acids but tryptophan and uracil, for plasmid selection. Cell numbers were determined using a Coulter counter (see Methods). The plates were incubated at 30°C for 4 days (Part a) or 35.5°C (Part b) for 6 days. Each strain was plated at three dilutions in columns. **Column I**, wild type UBC4 (*TRP1*) in combination with a *URA3* null plasmid (YEp24). **Column II**, the RAD6/UBC4 plasmid (*TRP1*) in combination with a *URA3* null plasmid (YEp24). **Column III**, the RAD6/UBC4 plasmid in combination with R3.2, a YEp24 plasmid with the entire 2 μ circle integrated. **Column IV**, the RAD6/UBC4 plasmid in combination with a plasmid expressing the *REP1* gene behind the *CUP1* promoter.

a. 30°C


Yeast Strain
I
II
III
IV
2x10^4
2x10^3
2x10^2
Cells Spotted

b. 35.5°C


Yeast Strain
I
II
III
IV
2x10^4
2x10^3
2x10^2
Cells Spotted

| A. Ratio of *URA3* Control Plasmid URA3 : TRP1 | | B. Ratio of *URA3* Library Plasmid 2 μ URA3 : TRP1 | |
|---|---|---|---|
| I. | 1.6 : 1 | I. | 5.53 : 1 |
| II. | 1.4 : 1 | II. | 4.73 : 1 |
| III. | 1.5 : 1 | III. | 5.50 : 1 |

TABLE 5.1

**Yeast 2 μ plasmid integration effects plasmid copy number.**

Shown are the calculated ratios of *TRP1* and *URA3* plasmids isolated from doubly transformed *ubc4/5Δ* strains in three individual experiments. In Part A, the ratio of standard *URA3* and *TRP1* high copy number plasmids was determined. The *URA3* control plasmid was YEp24, the library parental. The *TRP1* plasmid used in all cases was the RAD6/UBC4 plasmid. In Part B, the effect on copy number was determined for the *URA3* plasmid when the entire 6.3 kb 2 μ plasmid was integrated (plasmid R3.2). As detailed in the Materials and Methods section, the ratio of URA3 : TRP1 plasmids was determined by Southern analysis with non-radioactive labelled probes specific for either the *URA3* gene, or the *RAD6/UBC4* gene, followed by scanning densitometry of X-ray film to quantitate the signals generated by each probe. The two probes were used in succession on the same DNA samples and were corrected for differences in specific activity.

| | Growth Phenotypes of RAD6/UBC4 and Asp95 UBC4mutants in the *UBC4/5* deletion strain with or without the coexpression of *REP1*. | |
|---|---|---|
| | Strain | Vegetative growth (doubling time in h) |
| a. | *ubc4/5Δ +RAD6/UBC4+ null (URA3)* | 4.3 |
| | *ubc4/5Δ +RAD6/UBC4+ REP1* | 3.5 |
| b. | *ubc4/5Δ + Asp5 UBC4 + null (URA3)* | 4.5 |
| | *ubc4/5Δ + Asp95 UBC4 + REP1* | 3.2 |

TABLE 5.2

**The growth rate of the *ubc4/5Δ* strain expressing either the RAD6/UBC4 chimaera or the Asp95 UBC4 point mutant is increased by the coexpression of *REP1*.**

Shown is the calculated doubling rate at permissive temperature of yeast cells deleted for the *UBC4* and *UBC5* genes (*ubc4/5Δ*) and expressing one of two E2 mutants, in combination with a null *URA3*-based plasmid (YEp24) or with the REP1 high copy number plasmid containing the *CUP1* promoter. Doubling times were determined by counting equal aliquots of cells from each culture using a cell counter (see Materials and Methods). **Part a.** The doubling time of the *ubc4/5Δ* strain expressing the RAD6/UBC4 derivative in combination with a null plasmid or with *REP1*. **Part b.** The doubling rate of the Asp95 UBC4 derivative in combination with a null plasmid or with *REP1* expression. The Asp95 UBC4 mutant was expressed from a low copy number plasmid, and the RAD6/UBC4 chimaera was expressed from the *CUP1* promoter on a high copy number plasmid. Cells were grown in SD media containing the required nutrients at 30°C with the addition of copper.

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# CHAPTER 6. General Discussion and Conclusions

## 6.1 The identification and functional role of alternative Ub-Ub linkages *in vivo*.

The identification of alternative sites of Ub-Ub conjugation was accomplished by the construction and application of a series of novel Ub derivatives (Chapter 2). These investigations have demonstrated that three lysine residues within the Ub protein are suitable for Ub-Ub conjugate formation, at positions 29, 48 and 63. Our identification of three positions suitable for Ub-Ub conjugation both supports previous observations that Ub-Ub linkages other than the canonical K48 linkage could be formed *in vivo* and *in vitro* and extends these observations by identifying the nature of the linkages.

The role of the alternative linkages *in vivo* was analyzed. The ability of the Ub derivatives to complement for the stress *UBI4* gene revealed that the K29 and K48 linkages were not essential for the yeast stress response in our system, but that the K63 linkage played a vital role in stress resistance (Chapter 3). Loss of the K63 linkage (by replacement with arginine) lead to loss of stress resistance in the aforementioned yeast strain. Significantly, neither alternative linkage appeared to play a significant role in bulk protein degradation, an observation confirmed for the K63 linkage by Spence *et al.* (1995). However, it has very recently been shown that an artificial linear Ub-protein fusion substrate, when ubiquitinated by a K29 Ub linkage is degraded, and furthermore, that conversion of K29 to arginine dramatically stabilized the targeted protein (Johnson *et al.*, 1995). These results demonstrate that the K29 Ub-Ub linkage appears to have a role in protein degradation in the system used. In addition, Finley *et al.* reported that the loss of the K29 Ub-Ub linkage *in vivo* (by replacement with arginine) resulted in a slight growth defect (Finley *et al.*, 1994).

The K63 Ub linkage was later shown, by Spence *et al.*(1995) to be critical for yeast DNA repair after exposure to UV light, an observation our experimental system failed to detect. These combined results strongly suggest that the K63 Ub-Ub linkage is used in the specific Ub-dependent pathways of stress resistance and DNA repair and give rise to the intriguing possibility that different chain configurations function as discrete signals in unrelated processes. Questions remain, however, regarding how the selection of protein targets for alternative ubiquitination is made, the consequence(s) of alternative ubiquitination, and the mechanism whereby the linkage specificity is governed. These issues await further investigations for their resolution.

### 6.2 Linkage dependence of UBC4 and RAD6.

The E2 enzymes responsible for the formation of the alternative Ub-Ub linkages were identified (Chapter 3). Expression of the Ub derivatives in yeast strains deleted for E2 genes allowed us to determine if this loss was accompanied by the loss of a given linkage. Using this approach, it was found that the K29 Ub-Ub linkage required the Ub-conjugating enzyme UBC4 and presumably its close relative UBC5, and that the K63 Ub-Ub linkage required UBC4, RAD6 and UBC1, apparently with some codependence. The K48 Ub-Ub conjugate level was not affected by the deletion of any one E2 gene and therefore must be created by multiple E2 activities. The co-dependence of RAD6, UBC4, and the K63 Ub-Ub linkage in either the stress response, or DNA repair, suggests that distinct protein targets of the Ub system are ubiquitinated by the alternative Ub-Ub linkages by RAD6 or UBC4.

The notion that specific proteins are targeted by the E2s for alternative ubiquitination (in contrast to K48 multiubiquitination) was reinforced upon the observation that a RAD6/UBC4 chimaera and a UBC4 point mutant were defective in some, but not all, UBC4 functions *in vivo*, despite the retained ability of both to create the alternative linkages (Chapter 4). This result was unexpected, as UBC4 function in resistance to heat and amino acid analog stresses was assumed to be facilitated by the identical pathway of abnormal protein recognition and subsequent degradation (Seufert and Jentsch, 1990). Therefore, it may be that different protein factors must be targeted by UBC4 to overcome heat stress than are required for overcoming canavanine sensitivity. The possibility does exist, however, that different levels of UBC4 protein are required for the different stress conditions, and that the differences in stress resistance observed in our experimental system may reflect threshold levels of UBC4 activity for one stress versus another.

In summary, while not proven, it appears that the stress resistance function of UBC4, and the DNA repair function of RAD6, may require that certain cellular proteins be recognized and ubiquitinated by Ub chains which are composed of, or contain, the K63 alternative Ub-Ub linkage.

### 6.3. *Cis* and *trans*-acting factors affecting UBC4 functions.

Evidence that target recognition for alternative ubiquitination by UBC4 may rely on different E3s or *trans*-acting factors came from investigations of two E2 mutants. First, a UBC4 protein altered at the highly conserved serine 95 position was found to retain its ability to complement for heat stress resistance in a yeast strain deleted for the *UBC4/5* genes. However, this E2 was defective in its complementation of growth and canavanine resistance yet retained the ability to create the K29 and K63 Ub-Ub linkages. Secondly, a

RAD6 protein carrying two UBC4-specific amino acids (phenylalanine 63 and asparagine 80) was observed to have gained the ability to partially complement for growth rate and heat stress resistance, while remaining completely unable to complement for canavanine resistance. This same mutant had also gained the ability to create the K29 Ub-Ub conjugate previously formed only by UBC4. Together, these results suggest two hypothesis.

First, the Phe63, Asn80 and Ser95 residues may be involved in UBC4 function for growth and heat stress by their specific interactions with either target proteins or E3/*trans*-acting factors. Secondly, the ability of the UBC4 mutant and the RAD6 mutant to create both the K29 and K63 Ub-Ub conjugates, yet their inability to fully complement for UBC4 function implies that the ability to create the alternative linkage alone is not sufficient for UBC4 function, and instead that the alternative linkages must be targeted to appropriate proteins for function. Together, then, the physical associations between UBC4 and E3s/*trans*-acting factors may depend on the Phe63, Asn80 and Ser95 surface residues of UBC4.

Three additional facts suggest that at least two of the three UBC4 amino acids identified are involved in UBC4 growth function. First, the 95 and 80 positions are spatially adjacent (Figure 4.2), lending credence to the notion that these residues may be involved in the formation of a surface area necessary for protein interactions. Secondly, the RAD6 protein alone cannot provide UBC4-dependent growth without the introduction of the UBC4-Asn80 residue, despite the normal presence of the Ser95 residue, suggesting that the two residues work in concert. Lastly, the corresponding residue to Asp 80 was sufficient, when introduced to RAD6Δ, to complement for UBC4 growth function to a similar extent to that of the double RAD6/UBC4 chimaeric derivative, (Chantelle Gwozd, unpublished results) implying that the Phe65 residue of the RAD6/UBC4 is not necessary for chimaeric function. Together, these results suggest that the amino acid residues at position 95 and 80 of UBC4 may comprise a surface recognition cluster for protein interactions necessary for UBC4 growth function.

RAD6 function in DNA repair had been previously shown to depend on its physical associations with the *trans*-acting factor, RAD18 (Prakash, 1989). While there is no evidence to conclude that RAD18 affects the type of linkage formed by RAD6, it would be intriguing to directly test this possibility *in vivo*. An experiment using the Ubm derivatives (Chapter 3) in combination with a *RAD18* deletion strain may reveal that the K63 Ub-Ub conjugate levels are affected in this strain. UBC4, in contrast to RAD6, has not had E3s or *trans*-acting factors identified which are involved in its *in vivo* functions.

A genetic screen for UBC4-interacting proteins has yielded a potential candidate, REP1, encoded by the yeast 2 μ plasmid (Chapter 5). It is unknown how REP1 may affect

UBC4 function, or if UBC4 function in stress resistance is affected, as a *REP1* deletion strain exhibits no detectable phenotypes and no REP1 homologs have been found in higher eukaryotes. Our experiments have shown that *REP1* expression is able to affect the growth function of both UBC4 and UBC4-like derivatives. It was suggested by us that UBC4 may be recruited to the nuclear lamina by REP1, as REP1 becomes intimately associated with protein components comprising this structural matrix (Wu *et al.*, 1987). Therefore, it may be that the transport or recruitment of UBC4 to the nuclear periphery is somehow involved in its growth function, but this possibility has not been investigated and awaits further experimental work.

### 6.4. Are alternative Ub-Ub linkages behaving as different intracellular signals?

The investigations outlined in this thesis have provided insights into the Ub system and its role in the yeast stress response. The results obtained also provide new aspects for investigation of the Ub system *in vivo*. Future investigations regarding the possibility that the alternative Ub-Ub linkages function as distinct intracellular signals, apart from the K48 multiUb degradation signal, will be intriguing. A necessary initial step towards gaining a better understanding of the role of the alternative Ub-Ub linkages is to identify proteins targeted for alternative ubiquitination, perhaps by using the K29 and K63 specific antibodies whose generation is proposed in Appendix C. Once such targets are available, several specific questions can be addressed.

First, it can be determined whether the K29 and K63 Ub-Ub conjugates form homogenous multiUb chains as do the K48 chains, or if they are incorporated into preexisting chains to form mixed linkages. Next, the effect on the degradation rate, or stability, of natural proteins targeted by the K29 and K63 linkages can be investigated *in vivo*. Also lacking is our understanding of how these alternative linkages are recognized by the ceil, if in fact they are acting as discrete signals. Perhaps by using artificially created K29 or K63 Ub-Ub conjugates (Appendix C), it will be possible to determine if these alternative chain configurations can compete for binding to the protease subunits previously demonstrated to associate with the K48 multiUb chain (Deveraux *et al.*, 1995). If the alternative linkages are able to compete for subunit binding, this will reveal that the alternative linkages are in fact recognized by the same cellular machinery as the K48 multiUb chains. Conversely, if unable to compete with the K48 multiUb chain, the assumption may be true that the alternative linkages are structurally distinct, and that alternative ubiquitination of target protein may not necessarily result in proteasome interactions and subsequent degradation.

Lastly, it will be intriguing to identify protein factors which govern the linkage specificity and stress-protein target specificity of UBC4. For example, while the K63 Ub-Ub linkage created by UBC4 may be used to function in stress resistance, this functions is required only under specific conditions. It may be that proteins such as heat shock proteins, which are induced upon such stress conditions, may act as protein factors that govern the linkage specificity or protein recognition by UBC4 necessary for stress resistance function. For example, chaperonins may present misfolded damaged protein to the UBC4 enzyme in a recognizable form through their selective binding to these unfolded protein substrates (Mifflin *et al.*, 1994).

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## Appendix A. General Procedures

### A.1. Microbiological

#### A.1.a. Yeast manipulations

**i. Growth of Yeast cultures.** Culture media used in these studies were prepared according to Sherman *et al* (1986) and included (i) Yeast-Peptone-Dextrose (YPD; 2% (w/v) glucose, 1% (w/v) Bacto-Yeast Extract, 1% (w/v) Bacto-Peptone), (ii) Synthetic Defined (SD; 7 g yeast nitrogen base without amino acids and 20 g glucose per liter) supplemented with 20 mg/l of the following amino acids; lysine, uracil, histidine and tryptophan; (iii) Yeast Drop Out Media (as for SD media with the exception that all amino acids were included from sterile stock solutions with the exception of uracil and/or tryptophan); SD medium with canavanine (as for SD medium with the addition of 1.7 µg/ml canavanine from a 7.5 mg/ml stock solution). Bacto-Yeast Extract, Bacto-Peptone, Difco Bacto-Agar, and Bacto-Yeast Nitrogen Base were from Difco Inc. Plating medium was prepared by the inclusion of 2% (w/v) Difco Bacto-Agar in the medium. Plasmids were maintained in yeast strains by growth in Drop Out Media lacking uracil and/or tryptophan.

**ii. Permanent Storage of Yeast Strains.** Log-phase cells mixed with a sterile glycerol solution and rapidly frozen in liquid nitrogen can be stored for indefinite periods of time at -80°C. The strain can be frozen with transformed plasmids present. To revive the strain, frozen cells can be plated onto rich or selective yeast plates and incubated at 30°C until colonies develop. Freezer stocks were made as follows; 0.4 ml of YPD: glycerol (1:1 v/v) was filter sterilized and mixed with 0.6 ml of yeast culture in NUNC cryovial. After cooling on ice for 10 minutes, the mixture was then rapidly frozen in liquid nitrogen. Vials were then stored at -80°C.

**iii. Transformation of Plasmids into Yeast.** Yeast strains were transformed by either the PLATE transformation procedure (Elble, 1992), or by electroporation (Becker and Guarente, 1989) without bias.

For PLATE transformation, a mid-log phase culture growing in YPD was pelleted in a Damon centrifuge by centrifugation (3,000 x g, 5 min, 4°C). The pellet was resuspended in 20 ml sterile milliQ water, and 1 ml volumes were aliquotted into eppendorf tubes. The cells were again pelleted in a microfuge and the supernatant removed. For each transformation, 10 µl of herring sperm DNA (10 mg/ml, boiled and rapidly cooled before use) was mixed with the cells, followed by the addition of 5 µl of plasmid DNA(s). To each transformation tube, 500 µl of PLATE (4.06 g PEG 3000 [Sigma], 1.0 ml 1 M

lithium acetate, 100 µl Tris pH 7.5, 20 µl 0.5 M EDTA, milliQ water to 10 ml followed by filter sterilization) was added and mixed gently. After incubation overnight at room temperature, the cells were softly pelleted (1000 x g, 1 min), the PLATE solution removed by aspiration, and the pellet was resuspended in 100 µl of milliQ water. The resulting cell suspension was then plated on selective SD dropout plates lacking the appropriate specific nutrients for plasmid selection.

For electroporation, 100 ml cultures of mid log phase cells growing in YPD were pelleted, the supernatant decanted, and the cells resuspended in 12 ml sterile milliQ and placed in a culture flask. To this, 1.5 ml 10 x TE, 1.5 ml 1M Lithium acetate pH 7.5, and 375 µl of 1 M Dithiothreitol (DTT) was added and the culture incubated at 30°C with shaking for 15 minutes. The cells were then washed three times, first in 40 ml of ice cold sterile milliQ water, then in 3 ml of ice cold 1 M sorbitol (filter sterilized). The final cell pellet was resuspended in 75 µl of 1 M sorbitol. Each transformation used 20 µl of cells and 1 µl of plasmid DNA which was placed between the bosses of an electroporation cuvette. Electroporations were performed using the BRL Cell Porator, at 10 µF capacitance, fast charge and low resistance at 400 V. The cells were then removed from the electroporation chamber and put into 100 µl of cold 1 M sorbitol on ice for 30 minutes. Cells were then plated on minimal SD dropout plates containing 0.5 M sorbitol and lacking appropriate specific nutrients for plasmid selection.

**A.1.b. *E. coli* manipulations.**

**i. Plasmid propagation.** Plasmid propagation was carried out in MC1061 ( $F^-$*araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL* ($Str^r$) *hsdR2* ($r_k^-m_k^+$) *mcrA mcrB1*) (New England Biolab catalogue, 1995). *E. coli* was grown in Luria Broth (LB; 10 g/l Bacto-Tryptone, 5 g/l Yeast Extract, 5 g/l NaCl) or on LB Plates (LB containing 2% Bacto-Agar (w/v)) at 37°C. Plasmid maintenance was achieved by inclusion of ampicillin (50 mg/ml) in both or plate medium.

**ii. Permanent Storage of *E. coli* strains.** Permanents of *E. coli* strains with or without plasmids were frozen and regenerated essentially as for yeast strains, with the exception that for regeneration the *E. coli* cells were struck out onto LB or LB with antibiotic media. The freezer stocks were made as follows; 0.3 ml of LB: glycerol (1:1 v/v)-filter sterilized was mixed with 0.7 ml of *E. coli* culture, cooled on ice for 10 minutes and then rapidly frozen in liquid nitrogen. Vials were then stored at -80°C.

**iii. Transformation of Plasmid DNA into Bacteria.** Transformation was performed by the $CaCl_2$ method as previously described (Ausubel *et al.*, 1989) *E. coli* (MC1061 or BL21) were grown in LB medium until an $OD_{550}$ of 0.375 was reached and

then chilled on ice. The cells were pelleted by centrifugation (1600 x g, 5 min, 4°C), resuspended in 0.025 volumes of ice-cold $CaCl_2$ buffer (60 mM $CaCl_2$, 15% glycerol, 10 mM PIPES pH 7.0), and pelletted again by centrifugation. The cells were washed a second time with $CaCl_2$ buffer and resuspended in a small volume (1/200 of the original culture volume) of $CaCl_2$ buffer. Aliquots (200 µl) of the competent cell suspension were transferred to eppendorf tubes and frozen in liquid $N_2$ and stored at -80°C. For transformation, competent cells were thawed until just melting and put on ice. 100 µl cells was added to 1-15 µl DNA in an eppendorf tube, mixed, and put on ice for 20 minutes, transferred to 37°C for 5 minutes and placed again on ice for 20 minutes. The cells were plated directly onto LB plates that contained the appropriate antibiotics.

## A.2. DNA manipulations and Analysis

**A.2.a. Oligonucleotide synthesis.** Oligonucleotides for use in either sequencing or PCR were synthesized by the DNA synthesis and sequencing Facility in the Department of Biochemistry, University of Alberta.

**A.2.b. Isolation of plasmid DNA from *E. coli*.** Plasmid DNA for use in restriction enzyme analysis and subcloning was prepared using a modification of the alkaline lysis mini-prep procedure (Birnboim and Doly, 1979). A 3 ml portion of bacterial culture was centrifuged (17,000 x g, 5 sec) and the cells were resuspended in 0.1 ml Glucose-Tris-EDTA buffer (GTE; 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0). The cell suspension was mixed gently, by inversion, with 0.2 ml Lysis solution (0.2 M NaOH, 1% SDS) and incubated at 4°C for 5 minutes. After an addition of 0.15 ml potassium acetate solution (5 M acetate, pH 3.2), the tube contents were mixed by gentle inversion and centrifuged (17,000 x g, 5 minutes, 4°C). The supernatant was recovered and combined with 0.9 ml of ice-cold 95% ethanol prior to centrifugation (17,000 x g, 10 min, 4°C). To precipitate high molecular weight RNA, the pellet was dissolved in 0.1 ml TE buffer and the resulting solution was mixed with 0.1 ml 10 M LiCl and 0.05 ml of $CHCl_3$ and incubated at -20°C for 20 min. The solution was centrifuged (17,00 x g5 min, 4°C and plasmid DNA was precipitated from the aqueous layer by the addition of three volumes of 95% ethanol. The DNA was dissolved in TE buffer and stored at -20°C.

**A.2.c. Preparation of Plasmid DNA for Sequencing.** Plasmid DNA for sequence determination was prepared using an upscale version of the plasmid miniprep protocol. A single bacterial colony was inoculated into 250 ml of Terrific Broth (TB; 12 g bactotryptone, 24 g yeast extract, 4 ml glycerol into 900 ml. Separately autoclave 2.31 g $KH_2PO_4$, 12.54 g $H_2HPO_4$ in 100 ml. Combine before use) with ampicillin (50 µg/ml

final concentration) was inoculated with a single bacterial colony and incubated overnight in a 500 ml sterile flask. The next day, cells were centrifuged (20 minutes at 10,000 x g, 20 min, 4°C), in 250 ml centrifuge bottles in a Damon centrifuge. The supernatant was decanted and the cells resuspended in 20 ml of Solution 1 (2.5 ml 1 M Tris pH 7.5, 2 ml 0.5 M EDTA, 5 ml 20% glucose in 100 ml total volume). 40 ml of Solution 2 (10 ml of 10 % SDS, 20 ml 1.0 N NaOH in 100 ml total volume) were added and swirled to mix. 30 ml of 7.5 M ammonium acetate (Sigma), was then added and the mixture was inverted 3x to mix followed by incubation on ice for 15 minutes. The mixture was filtered through triple layered cheesecloth into fresh centrifuge bottles, and then centrifuged (10 minutes, 10,000 x g, 4°C). To the supernatant, 46 ml of isopropanol was added and followed by incubation at -20°C for 20 minutes. To pellet the DNA, the solution was centrifuged (10,000 x g, 10 min, 4°C), the supernatant was discarded and the pellet and bottle walls were dried by gentle aspiration. The DNA pellet was resuspended in 2.5 ml of 10 x TE and transferred equally to three eppendorf tubes. RNA was removed by adding 20 µl of 20 mg/ml RNAse stock to each tube and incubating at 37°C for 30 minutes. The DNA solution was extracted with phenol/chloroform/isoamylalcohol (ratio of 25:24:1) until the white interface disappeared. Each tube was then filled with isopropanol, incubated at -20°C (15 min) and centrifuged (17,000 x g, 10 min, 4°C) to pellet the DNA. The three DNA pellets were resuspended in 400 µl milliQ water total and pooled into 1 tube. The DNA concentration of the purified DNA sample was then determined by fluorometry at pH 12. DNA sequences were determined using an automated DNA sequencer (Applied Biosystems) operated by the Department of Biochemistry DNA Sequencing and Synthesis Facility at the University of Alberta. 200 ng/µl DNA template was used with 3.2 pmol forward and reverse primers. The sequencing primers were either chemically synthesized or available Universal primers were used. The double stranded DNA sequencing used fluorescent dye terminators, based on the sequencing method by Sanger et al.

**A.2.d. Isolation of Plasmid DNA from *S. cerevisiae*.** Plasmid DNA was isolated from yeast by the method of Baker and Schatz (1987). Portions (5 ml) of yeast culture were centrifuged (2,000 x g, 5 min) and the cells were washed with 1 ml of yeast lysis buffer (1 M Sorbitol, 60 mM EDTA 0.1 M $NaH_2PO_4$ pH 7.0) prior to resuspension in 0.2 ml lysis buffer that contained 2 mg/ml Zymolyase 5000 (Kirin Brewery) and 28 mM dithiothreitol (DTT). The cell suspension was incubated at 37°C for 60 min and then cells were lysed by the addition of 0.4 ml of 0.2 M NaOH/1% SDS and incubated at 4°C for 5 min. The lysed cells were mixed with 0.5 x volume of 3.0 M $CH_3COOK$ (pH 4.8) and incubated (4°C, 5 min) prior to centrifugation (9,000 x g, 5 min). The supernatant was recovered and mixed with 2.5 volumes of 95% ice cold ethanol prior

to centrifugation (9,000 x g, 10 min). The plasmid-containing pellet was resuspended in TE buffer and stored at -20°C.

**A.2.e. Enzymatic Treatment.**

**i. Polymerase Chain Reaction (PCR): General conditions**

PCR reactions were routinely carried out in 100 µl volumes using a Perkin Elmer Cetus Thermal Cycler 480 model. All PCR reactions consisted of 10 µl of 10x PCR Reaction Buffer (BRL), 16 µl of dNTP mix (2 mM stock), 1 µl template DNA (from miniprep), 1nM each of appropriately designed primers, 0.5 µl Taq Polymerase (BRL) and brought to 100 µl total volume with milliQ water (Saiki *et al.*, 1988). After mixing, the reactions were overlaid with heavy mineral oil to prevent evaporation during temperature cycling. PCR reactions were carried out for 30 cycles using annealing and melting temperatures calculated for each primer pair.

Following PCR, samples were concentrated by vortexing 200 µl of chloroform into the PCR mineral oil mixture. The resulting emulsion was then centrifuged for 2 minutes and the DNA aqueous phase (top layer) was transferred to a fresh tube.. To this, 5 µl of 3M sodium acetate pH 5.2, and 300 µl of 95% ethanol were added and mixed. The tube was then incubated at -80°C for 1 hour to precipitate the DNA. The DNA was pelleted by centrifugation (17,000 x g, 15 min). The supernatant was removed, and the DNA pellet was washed with 70% ethanol and dried on the bench top. The DNA was then resuspended in a convenient volume (50 to 100 µl) of 1 x TE (10 mM Tris, pH 7.5, 0.1 mM EDTA, pH 8) or milliQ water.

**ii. Restriction enzyme digestion of plasmid DNA.** DNA fragments and PCR products were digested generally as follows. The reaction mixture contained 0.1 volumes of the appropriate commercially available restriction buffer (10x concentration, BRL), 0.1 volume of DNA solution, 0.05 volumes of restriction enzyme stock, and 0.75 volumes of sterile milliQ water. Restriction digests were carried out in 15 µl reactions for at least 1 hour at the specified temperature. Following digestion, 3 µl of 5x DNA loading buffer (for 50 ml: 15 g glucose, 500 µl 100x TE, 0.125 g bromophenol blue) was mixed in with 0.5 µl RNAse (20 mg/ml stock) and the entire volume loaded onto either an agarose gel or a polyacrylamide gel.

**iii. Ligation of DNA fragments.** Ligation of DNA fragments was performed in a 15 µl ligation reaction, which included 3 µl of 5x ligation buffer (Gibco/BRL), 2 µl of T4 DNA Ligase (Gibco BRL), 5 µl DNA and 5 µl milliQ water reaction mixtures were incubated overnight at 16°C in the Perkin Elmer Cetus PCR block.

**iv. Dephosphorylation of DNA ends.** Dephosphorylation of DNA ends was carried out by adding 1 µl of Shrimp Alkaline Phosphatase (United States Biochemical)

directly to the restriction enzyme reactions and was inactivated by heating to 65°C for 10 minutes.

**A.2.F. Electrophoretic methods.**

**i. PCR and fragment analysis.** Analysis of DNA products of restriction enzyme digests and PCR reactions were performed by electrophoretic separation of samples on 1% agarose gels. Agarose gels ranging from 1% to 2% were used to separate DNA fragments depending on the size of fragments being analyzed. For a 1% gel, 1 gram of agarose (Gibco BRL) or low melting point agarose (LMP, Gibco BRL) was heated in 100 ml of 1x TAE (from 50x stock in 1 liter: 242g Tris, 57.1 ml glacial acetic acid, 37.2g NaEDTA·$2H_20$, pH 7.6) until the agar was melted. The agarose concentration of the gel was increased to 2% when DNA fragments less than 200 bp were expected. λHindIII and 123bp ladder (Gibco BRL) molecular weight standards were used to evaluate fragment size. 1 liter of 1x TAE had 15 µl of ethidium bromide included in the running buffer for detection of DNA under UV light.

For DNA fragments in the size range of 60-200 bp, electrophoretic separation was preferred using a polyacrylamide gel (8%). The gel was made as follows; 9 ml of 45% acrylamide stock solution, 1 ml of 50x TAE (no ethidium bromide), 0.5 ml 10% APS (1 g ammonium persulphate in 10 ml milliQ water) and milliQ water to 50 ml total was mixed and degassed on a vacuum line. 15 µl of TEMED (Sigma) was added for polymerization. The gel was cast between glass plates with 1.5 mm width spacers. DNA samples were mixed with 5 x DNA loading buffer and 1% TAE without ethidium bromide was used as the running buffer. The gel was run at 200 volts until the dye front was at the bottom of the gel. The gel was then soaked in 1x TAE with ethidium bromide for 15 minutes and the DNA was visualized using a UV light (254 nm) transilluminator.

**ii. DNA fragment purification**

Gel purification of DNA fragments was performed by electrophoretic separation on a 1% low melting point (LMP) agarose gel in TAE buffer (Favre, 1992). The band of interest was cut out in a minimal volume of agarose. 2.5 x volume of 50 mM NaCl was added and the agarose melted at 65°C for a maximum of 10 minutes. After the agarose was melted, an equal volume of buffered phenol was added and vortexed briefly. After centrifugation (17,000 x g, 2 min) the supernatant was transferred to a fresh tube. The phenol step was repeated until no interface was visible. To the final supernatant, an equal volume of *n*-butanol was added to the aqueous phase, vortexed and centrifuged (17,000 x g, 2 min). The aqueous phase (bottom layer) was transferred to a fresh tube and 3 volumes of chilled 95% ethanol was added. The DNA was precipitated by incubation at -80°C for 30

minutes, followed by centrifugation (17,000 x g, 15 min). DNA pellets were resuspended in 10-20 µl of TE buffer and stored at -20°C.

**iii. Oligonucleotide purification.** Synthetic oligonucleotides were purified by preparative electrophoresis as follows: Gel preparation- for 100 mls, 50 ml 45% polyacrylamide:2.25% Bis (90 g acrylamide, 4.5 g bisacrylamide), 20 ml 5x TBE (1 liter 10x stock; 108g Tris, 55g Boric acid, 40 ml EDTA pH 8 ), and 48 g urea were combined. The solution is dissolved by heating in 37°C water bath. To polymerize the gel, 0.5 ml 10% APS, and 20 µl TEMED were added before pouring into the gel mold). The acrylamide gel mold was made using 3 mm spacers. The running buffer was 1x TBE (for 1 liter of 10x stock: 108 g Tris HCl, 55 g boric acid, 40 ml of 0.5 M EDTA, pH 8). The gel was prerun for 30 minutes at 250 Volts. Desiccated oligonucleotide DNA was dissolved in 250 µl milliQ water and heated for 20 minutes at 50°C. Undissolved material was removed by centrifugation (10,000 x g, 5 min). The 250 µl DNA sample was diluted in an equal volume of 5x DNA loading buffer. 125 µl was loaded per lane and the gel was run for at least 2 hours at 250 V for best separation. After separation, the top glass plate of the gel mold was removed, and the gel was put under a UV light to visualize the DNA. DNA exposure to UV light was minimized to prevent UV damage. The band of interest was cut out (highest, darkest band per lane) and the gel piece was crushed by injection through a 5 ml syringe. The crushed gel was collected in a 15 ml Falcon tube and 2.5 ml milliQ water was added, followed by slow agitation overnight at 37°C to elute the DNA. The liquid from the filtered solution was then dried under vacuum. 1.2 ml 95% ethanol was added to each tube to wash the DNA pellet, and repeated four times. The last ethanol supernatant was discarded and the DNA pellet was dried down. The concentration of the oligonucleotide was determined by spectrophotometry at 260 nm by diluting 5 µl of the oligonucleotide sample into 1 ml milliQ water. The µg/ml DNA was calculated using the following conversion.

$OD_{260}$ x 200 (dilution factor) x 30 µg/ml/OD. = µg/ ml DNA.

### A.3 Protein Purification and analysis.

**A.3.a. SDS Polyacrylamide gel electrophoresis (PAGE).** Proteins were separated by SDS PAGE essentially as described (Laemlli, 1970) but with slight modifications. In all cases protein samples were electrophoresed on an 18% acrylamide, 0.09% bisacrylamide separating gel that was prepared by combining 15 ml of 36% acrylamide/0.18% Bis (500 ml: 180 g acrylamide, 0.9 g bisacrylamide [Sigma]), 15 ml 4x Tris pH 8.8 (500 ml: 91 g Tris base, HCl to pH 8.8 ), and 300 µl 10% APS (ammonium

persulphate) were combined in a side arm flask and degassed under vacuum for 5 minutes. 300 µl of 10% SDS and 10 µl TEMED (Gibco BRL) were then added. MilliQ water was gently layered on top of the unset gel to ensure a smooth interface. Once polymerized, the stacking gel was layered on top.

The stacking gel was prepared by combining 1.2 ml 30% acrylamide (500 ml: 150 g acrylamide, 4 g Bis, final solution is filtered), 1.25 ml 8x Tris pH 6.8 (100 ml: 12.1 g Tris base, HCl to pH 6.8), 50 µl 10% APS, 100 µl 10% SDS, to a final volume of 10 ml. Following removal of the water from the separating gel, 10 µl of TEMED was added for polymerization to the stacking mixture which was then layered over the separating gel followed by the insertion of a 20 well comb. Once polymerized, the comb was removed under running water. The gel was then assembled onto the electrophoresis apparatus (BRL) and the upper and lower reservoir chambers were filled with running buffer (4 liters of 5 x stock: 60.4 g Tris base, 288 g glycine, and 20 g of SDS, and milliQ water to 4 liters). Samples were routinely electrophoresed at 250 V.

b. Sample concentration and preparation. The $OD_{600}$ of the yeast cultures was taken before pelleting the cells and the sample was prepared for SDS PAGE analysis by resuspending the cells directly in 2x electrophoresis load mix. The volume of load mix added was calculated as follows:

$OD_{600}$ x volume of culture (ml) x 5 = µl of 2 x sample buffer. Protein samples of whole cell lysate from yeast cells overexpressing Ub derivatives were boiled for 10 minutes and centrifuged (17,000 x g, 5 min) to remove cellular debris. Sample supernatants were electrophoresed on an SDS-polyacrylamide gel. For all Western samples, 3 µl to 10 µl of whole cell lysate in 2 x SDS loading buffer was loaded per well and was sufficient material for immunoblot detection. The gels were cast between glass plates using 0.5 mm spacers. Low Range SDS-PAGE prestained protein molecular weight standards were from BioRad.

**A.3.b. Detection**

**i. Coomassie detection** of proteins following SDS PAGE. The acrylamide gel was stained with Coomassie Brilliant blue by immersing the gel in 50 ml of stain (25%v/v methanol, 10% acetic acid, 0.05% w/v Coomassie Brilliant blue) for at least 30 minutes with agitation. To destain, the gel was then transferred to 100 ml destain solution (25% v/v methanol, 10% acetic acid) and agitated for at least 1 hour.

**ii. Western analysis.** After protein separation by SDS PAGE, proteins were transferred to an Immobilon-P membrane (Polyvinylidine difluoride [PVDF]) using an electroblotting apparatus (Idea Scientific Company). The protein was transferred in Transfer Buffer (2 liter transfer solution; 6.06 g Tris, 28.8 g glycine, 400 ml methanol, milliQ water to 2 liters) at a constant 20 Volts until the amperage reached 1.00. The

apparatus was disassembled, and the membrane was blocked by adding 25 ml of 20% FCS (fetal calf serum) in 1 x HNN (10 x stock: 500 mM Hepes pH 7.5, 500 mM NaCl, 0.2% sodium azide) to the membrane in a Pyrex dish for 1 hour, rocking, at room temperature. After blocking, the solution was kept and saved for further use.

**a. anti Myc Western analysis.** The anti myc antibody (2:3 dilution of 9E10 cell supernatant in 1x HNN with 20% FCS) was allowed to adsorb to the Immobilon-P membrane by incubating 20 ml of the antibody solution with the blocked PVDF membrane in a Pyrex dish (at 10°C) with gentle rocking overnight. The antibody solution was removed and saved for subsequent Westerns. The blot was washed three times with 20 mls of 1 x Tris buffered saline plus Tween-20 (TBS + Tween; 10 mM Tris-HCl pH 7.5, 0.9% NaCl, 0.05% Tween-20) for 10 minutes per wash. The membrane was then incubated in 15 ml of TBS+Tween containing 7.5 µl of secondary antibody for two hours. Myc epitope containing proteins were visualized by either of two methods, Alkaline Phophatase color development, of Chemiluminescence.

a (i). Alkaline phophatase (AP) Color Development. The secondary antibody for color development was biotinylated goat anti mouse (BioRad) antibody which was allowed to adsorb for 2 hours with gentle rocking at 10°C. Streptavidin-(AP) conjugate was then added at a 1000 fold dilution and incubated for 1 hour at 4°C with rocking. The color reaction was carried out as follows: the PVDF membrane was thoroughly washed in TBS +Tween, blotted semi dry, and transferred to a fresh Pyrex dish containing 15 ml of color solution (15 ml AP buffer [100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl], 100 µl NBT [Promega], 50 µl BCIP [Promega]). After sufficient color development, the color reaction was stopped by transferring the blot to a dish containing Stop solution (20 mM Tris pH 8, 5 mM EDTA).

a (ii). For Chemiluminescent detection (ECL, Amersham) the membrane was treated as above except that the biotinylated secondary antibody was substituted with goat-anti mouse horseradish peroxidase conjugated IgG. Following washing to remove the secondary antibody, the membrane was treated with Amersham ECL detection reagents according to company specifications followed by visualization by autoradiography.

**b. anti Ub Western Analysis.** After blocking with 20% FCS in 1 x HNN, 15 µl of rabbit -anti-Ub antibody in 15 ml of 20% FCS in 1x HNN was added and allowed to incubate overnight at 10°C with gentle shaking. The blot was washed three times for 10 minutes with 1 x ANT (10 x stock: 500 mM Hepes pH 7.5, 500 mM NaCl, 0.2% sodium azide) with 0.05% Tween-20 (Sigma). For those Westerns developed using the (AP) Color reaction, the anti rabbit IgG-AP secondary antibody was added at a 1:1,000 dilution in 1x ANT containing 0.5% Tween (15 ml final), and allowed to adsorb for 2 hours at 10°C with

gentle shaking. Color development was carried out as described above. Chemiluminescent detection was performed as described above using the goat anti rabbit HRP conjugate.

iii. Autoradiography. Proteins in yeast cultures that had been grown in the presence of radiolabelled amino acids ([$^{35}$S]methionine or [$^{14}$C]lysine, ICN Biomedicals, Inc.), and had the radiolabelled protein separated by SDS PAGE were analyzed by autoradiography. The acrylamide gels or radioactive Immobilon-P membrane were exposed to X-ray film after impregnation with Enhance and held at -80°C for a suitable length of time for detection. X-ray film was developed using an automated KODAK X-ray developing machine, in the Department of Biochemistry, University of Alberta.

Fetal Calf Serum was obtained form Gibco Laboratories. Immobilon-P membrane was from Amersham; antibodies (rabbit anti-Ub, biotinylated goat anti mouse, streptavidin-alkaline phosphatase conjugate, goat anti mouse horse radish peroxidase conjugated IgG, and rabbit IgG-alkaline phosphatase complex) were obtained from BioRad Laboratories Inc.; 9E10 cell line was a gift from G. Evan (1985).

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## Appendix B.

## Details of Ub derivative construction.

This appendix provides the details of the PCR mutagenesis and the cloning strategy used for the construction of the different Ub derivatives in this thesis. The starting genetic material was either a wild type Ub gene cassette (Ub) or a Ub gene cassette mutated so that the expressed Ub protein carried an arginine mutation at position 48 (R48Ub) (Ecker *et al.*, 1987). All other mutations introduced into the Ub protein are described in the following pages.

### B.1. Construction of double lysine (K) to arginine (R) UbΔ acceptor derivatives.

The lysine codon in each instance was converted to arginine (AGA) by site directed mutagenesis using the Polymerase Chain Reaction (PCR) (Figures B.1 and B.2). A series of oligonucleotides were designed to specifically mutate individual lysine codons to arginine codons. Each oligonucleotide retained the restriction sites present in the original, wild type Ub gene cassette to facilitate the reconstitution of full length Ub genes (Table B.1). Each Ub derivative gene created through PCR varied from the others in the position of the second arginine mutation other than at position 48. These PCR products were used to replace a yeast Ub gene cassette deleted for the final two glycine residues (Gly75, Gly76, referred to as UbΔ), resulting in Ub genes that had double arginine replacements at lysine positions and also were deleted for the C-terminal sequences. These UbΔ derivatives were placed on a high copy *TRP1* based yeast vector behind the highly inducible *CUP1* promoter. Confirmation that the wild-type UbΔ gene had been replaced with the Ub derivative PCR products was carried out initially by restriction analysis where possible. DNA sequence alterations introduced by the codon changes also introduced and removed unique restriction sites. In general, the mutation of K48 to R48 introduced an *Acc*1 site and the mutation of K29 to R29 introduced an *EcoR*1 site. *EcoR*1 digestion of a R29 Ub derivative will release a 118 base pair fragment, and a *BglII*/*Acc*I digest of an R48 Ub derivative will release a 144 base pair fragment, in contrast to a 189 base pair fragment released by the same digest of K48 Ub. The DNA sequence of all Ub derivative genes was further confirmed by double stranded DNA sequencing of the entire genes.

**B.2. Single Lysine UbΔ acceptor derivatives were constructed in a multistep process.**

The details of the methods used to create the series of Ub acceptor derivatives with all, or all but one of the seven lysines in Ub converted to arginine are outlined in Figures B.3, B.4, B.5 and B.6. The mutations were introduced by PCR using oligonucleotides designed to generate fragments of the Ub gene cassette with multiple lysine-to-arginine conversions. The original restriction sites present in the wild type Ub gene cassette were maintained to allow for the exchange of Ub gene fragments. This was facilitated by the presence of scattered, unique restriction sites present throughout the gene cassette, restriction sites which were likewise incorporated into the PCR fragments by their presence on the oligonucleotides which were used. Each Ub derivative varied from the next only in the position of the single remaining lysine. In general, lysine codons were replaced by the AGA codon of arginine by PCR. The construction of these derivatives was a multistep process, requiring the juxtaposition of various mutated regions of the Ub gene cassette to form the full length Ub genes. In several cases, 5' fragments of the Ub gene were ligated together to the 3' end of previously available Ub derivatives by a common restriction site and successful ligation was selected for by amplification using PCR with oligonucleotides specific for the full length Ub gene. The full length Ub derivatives were introduced onto a *TRP1*-based yeast plasmid behind the *CUP1* copper-inducible promoter by replacing Ub genes deleted for the C-terminal glycines (UbΔ). As for the double arginine UbΔ derivatives (Figures B.1 and B.2), the mutations at positions 29 and 48 introduces unique DNA restriction sites into the coding sequence that could be analyzed by acrylamide gel electrophoresis to confirm that the mutations had been introduced. The sequences of the Ub gene derivatives were further confirmed by double stranded (ds) DNA sequencing.

**B.3. Construction of Ubm derivatives with K-to-R replacements at any of positions 29, 48 or 63.**

The construction of Ub derivatives with C-terminal Myc-epitopes is detailed in Figure B.7. The previously constructed UbΔ derivatives with double arginine mutations including R48 made it possible to exchange these derivitized Ub cassettes from their UbΔ context into a cassette fused at the C-terminus to the Myc epitope sequences in an identical manner as the conversion of the single lysine UbΔ derivatives to Ubm derivatives. This resulted in the creation of Ubm derivatives with the identical DNA sequence as their UbΔ counterparts with the exception of the presence of the Myc epitope at the C-terminus. However, the R29,R49,R63 Ubm (RRR.Ubm) had not been previously made in any

context, making it necessary to splice together portions of Ub cassettes that had been previously mutated.

### B.4. Construction of full length Ub derivatives with K-to-R replacements at any of positions 29, 48 and 63.

For phenotype analysis of the three alternative Ub-Ub linkages (Chapter 3), Ub derivatives were provided as full length protein products so that they could function as donors and acceptors, *in vivo*, in Ub-Ub conjugate formation. The strategy for the creation of these Ub derivatives is detailed in Figure B.8.

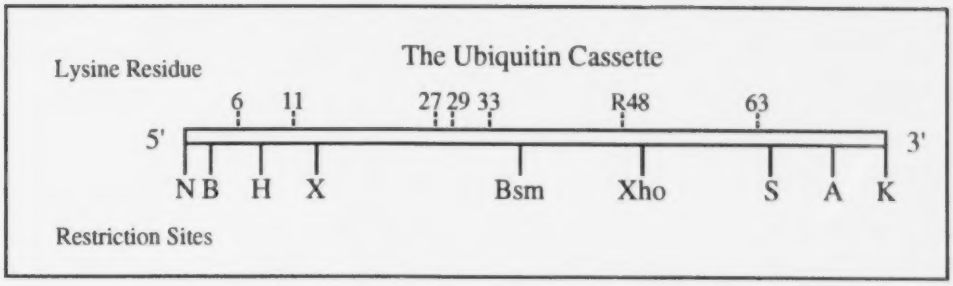
FIGURE B.1

**PCR and Cloning strategy for the construction of R6R48UbΔ, R11R48UbΔ and R63R48UbΔ.**

A schematic diagram of the R48 ubiquitin gene cassette is shown and drawn to scale. The seven lysine (K) positions are numbered and their positions are shown relative to the restriction sites in the Ub cassette.

I. The introduction of arginine (R) codons (AGA) for lysine codons was facilitated by PCR. The DNA template for PCR reactions A, B and C was a Ub cassette differing from wild type only by the presence of an arginine (R) AGA codon at position 48. The oligonucleotide primers and the sites of mutation in the three PCR products are aligned with the Ub cassette above for reference. **PCR A**. the 5' oligonucleotide MEL33 was used to introduce an R codon at position 6. **PCR B**. The 5' oligonucleotide MEL34 was used to introduced an R codon at position 11. For both PCR A and B the 3' primer was MEL39. **PCR C.** The 3' primer used to introduce an R codon at position 63 was MEL 38. The 5' primer was MEL32. MEL39 and MEL32 do not introduce any sequence alteration into the Ub cassette. PCR reactions were carried out for 30 cycles with an annealing temperature of 55°C. PCR products A and B were 240 bp and the PCR C product was 200 bp.

II. The PCR products were used to replace a wild type Ub cassette lacking the terminal two glycine residues (Gly75 and Gly76, denoted as UbΔ). *Bgl*II-*Sal*I digested PCR products were individually ligated to the large *Bgl*II-*Sal*I fragment from the wild type UbΔ plasmid. The resulting Ub derivatives were converted to UbΔ cassettes with two lysine mutated to arginines, one of which is always at position 48. The restriction sites in the Ub cassette are as follows: N, *Nde*I; H, *Hpa*I; B, *Bgl*II; X, *Xba*I; Bsm, *Bsm*I; Xho, *Xho*I, S, *Sal*I; A, *Afl*II; K, *Kpn*I.




I. Introduction of lys-to-arg mutations.

**PCR A. R6, R48 Ubiquitin**

**PCR B. R11, R48 Ubiquitin**

**PCR C. R48, R63 Ubiquitin**

II. Creation of UbΔ Derivatives

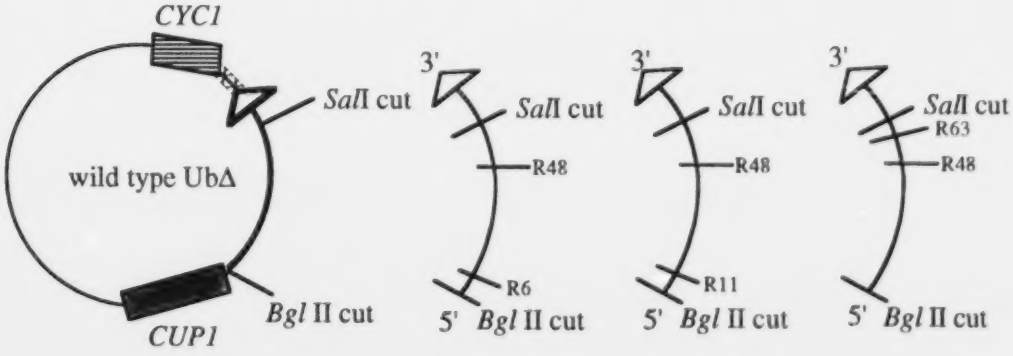


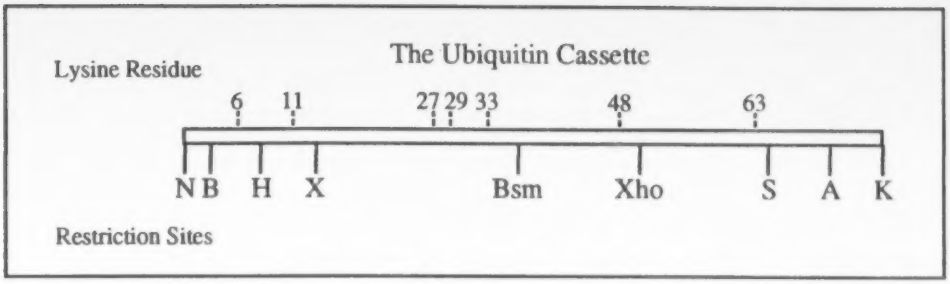
FIGURE B.2

**PCR and Cloning strategy for the construction of R27R48UbΔ, R29R48UbΔ and R33R48UbΔ.**

A schematic diagram of the wild type ubiquitin gene cassette is shown and drawn to scale. The seven lysine (K) position are numbered and their position is shown relative to the restriction sites in the Ub cassette.

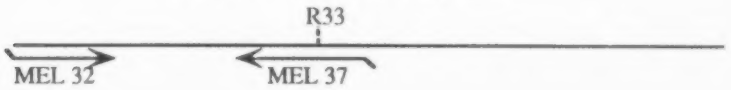
I. The introduction of arginine (R) codons (AGA) for lysines codons was facilitated by PCR. The DNA template for PCR reactions D, E and F was a wild type Ub cassette . The oligonucleotide primers and the sites of mutation in the three PCR products are aligned with the Ub cassette above for reference. **PCR D.** the 3' oligonucleotide MEL37 was used to introduce an R codon at position 33. **PCR E.** The 3 oligonucleotide MEL35 was used to introduced an R codon at position 27. **PCR F.** The 3' primer used to introduced R codon at position 29 was MEL 36. The 5' primer in all instances was MEL32. MEL32 does not introduce any sequence alteration into the Ub cassette. PCR reactions were carried out for 30 cycles with an annealing temperature of 55°C. PCR products D, E and F were 112 bp.

II. The PCR products were used to replace an R48 Ub cassette lacking the terminal two glycine residues (Gly75 and Gly76. denoted as UbΔ). This cassette differed from wild type only in the replacement of lysine sequences at position 48 with an arginine codon. *Bsm*I digested PCR products were individually ligated to the large *BamH*I-*Bsm*I fragment from the R48 UbΔ plasmid. A portion of the ligation reaction was used as a template in a PCR reaction to select for ligated, full length Ub genes using the 5' primer MEL32 and the 3' primer MEL39. These 240 bp PCR fragments were cut with *Bgl*II-*Kpn*I and replaced the *Bgl*II-*Kpn*I wild type Ub cassette lacking the C-terminal glycine residues (Gly75 and Gly76) present on a *TRP1* yeast vector. The resulting Ub derivatives were thus converted to UbΔ cassettes which had two lysine converted to arginines, one of which is always at position 48. The restriction sites in the Ub cassette are as follows: N, *Nde*I; H, *Hpa*I; B, *Bgl*II; X, *Xba*I; Bsm, *Bsm*I; Xho, *Xho*I, S, *Sal*I; A, *Afl*II; K, *Kpn*I.

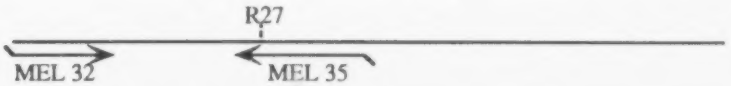



I. Introduction of lysine-to-arginine mutations by PCR.

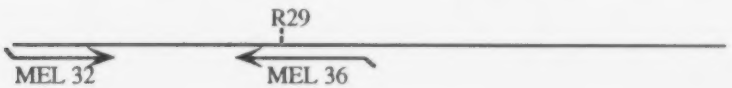
**PCR D. R33, R48 Ubiquitin**




**PCR E. R27, R48 Ubiquitin**




**PCR F. R29, R48 Ubiquitin**




II. Creation of UbΔ Derivatives

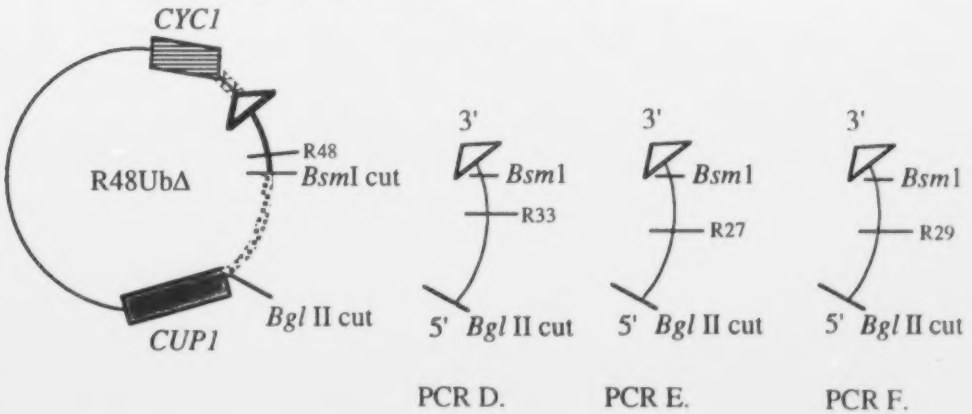


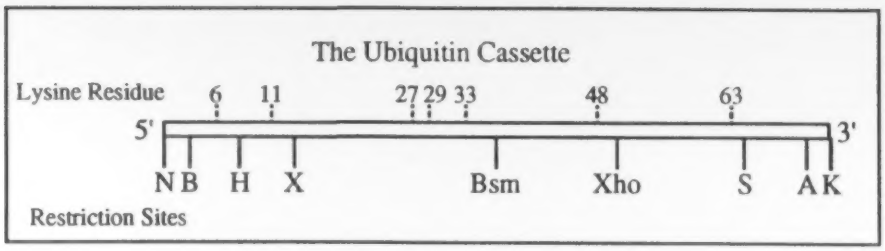
FIGURE B.3

**STEP 1 in the multistep PCR strategy designed for the construction of single lysine derivatives of Ub: Introduction of 5' mutations**

A schematic diagram of the wild type ubiquitin gene cassette is shown and drawn to scale. The seven lysine (K) position are numbered and their position is shown relative to the restriction sites

The first step in the construction of Ub derivatives mutated to possess a single lysine residue of the seven normally resent was to introduce lysine-to-arginine conversions at the first five lysines encoded by 5' Ub gene cassette sequences. Each PCR product differed from the next only in the position of the single remaining lysine residue present in the 112 bp products. Mutations were introduced by PCR using pairs of oligonucleotides designed to introduce numerous arginine codons at a time. The template used was a wild type Ub cassette, and the PCR reactions were for 30 cycles with an annealing temperature of 55°C.

A schematic of the Ub cassette is shown drawn to scale, with the position of the seven lysines relative to the unique restriction sites as indicated. PCR products #1a to #6a are shown aligned with the cassette and the direction and identity of each pair of PCR primers is as shown. The resulting combination of lysines (K) and arginines (R) for each PCR reaction is denoted by the aligned representative letters (K or R) with the Ub cassette. The restriction sites in the Ub cassette are as follows: The restriction sites in the Ub cassette are as follows: N, *Nde*I; H, *Hpa*I; B, *Bgl*II; X, *Xba*I; Bsm, *Bsm*I; Xho, *Xho*I, S, *Sal*I; A, *Afl*II; K, *Kpn*I.




**PCR #1. Total N-terminal lysine-to-arginine conversion.**

**PCR #2a. Lysine (K) 6 remaining**

**PCR #3a. Lysine (K) 11 remaining**

**PCR #4a. Lysine (K) 27 remaining**

**PCR #5a. Lysine (K) 29 remaining**

**PCR #6a. Lysine (K) 33 remaining**

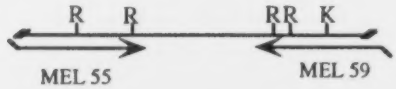


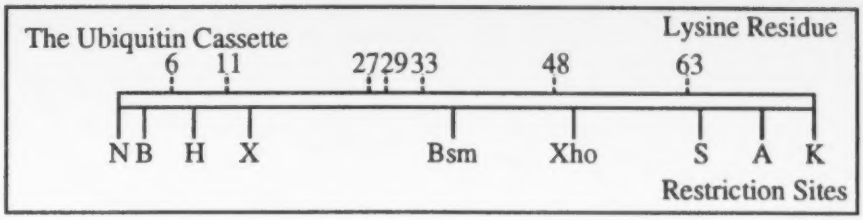
FIGURE B.4

**STEP 2 in the multistep PCR strategy designed for the construction of single lysine derivatives of Ub: Creation of K48UbΔ and K63UbΔ.**

The second step in the construction of Ub derivatives mutated to possess a single lysine residue of the seven normally present was to piece together the 5' mutated fragments generated in STEP 1 with 3' fragments of previously existing Ub cassettes. The juxtaposition of Ub cassette fragments was selected for by PCR. In this manner, Ub genes were created that had a single lysine at position 48 (K48Ub) and position 63 (K63Ub).

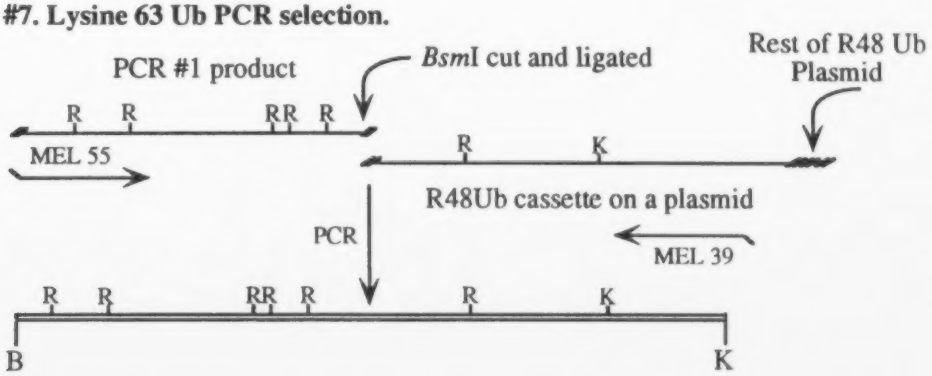
**PCR#7**. A yeast plasmid with the R48 Ub cassette was digested with *Bsm*1 and *BamH*I to release the 3' fragment of the R48Ub cassette from uncut vector. DNA generated by PCR #1 (all five 5' lysines converted to arginine) was also cut by *Bsm*I and ligated with the cut *Bsm*I-*BamH*I vector. A portion of the ligation reaction was used as template in a PCR reaction using oligonucleotides to amplify the full length Ub cassette without introducing any sequence changes. **PCR #8**. A yeast plasmid was first made which had an R63 Ub cassette. To make this cassette, a wild type Ub cassette was used as template in a PCR reaction with MEL32 and MEL38. This resulted in the introduction of an arginine codon (AGA) at position 63 only. The *Sal*I cut PCR product was ligated with a *BamH*I-*Sal*I cut wild type Ub gene cassette and successful ligations were selected for by PCR. After cloning this cassette into a yeast *TRP1* plasmid, a portion of the R63 cassette was removed by digestion with *Bsm*I and *BamH*I the remaining vector was ligated to the *Bsm*I digested PCR #1 DNA. As for PCR #7, successful ligations were selected by PCR. PCR reactions #7 and #8 used MEL55 as a 5' primer and MEL39 as the 3' primer. Both reactions were for 30 cycles and the annealing temperature was 38°C.

The K63 and K48 Ub cassettes were converted to a UbΔ context in an identical manner as the double arginine mutants were. Briefly, *Bgl*II-*Sal*I digested PCR products were used to replace the internal sequences of a UbΔ wild type cassette. A schematic of the Ub cassette is shown drawn to scale, with the position of the seven lysines relative to the unique restriction sites as indicated. PCR products #7 and #8 are shown aligned with the cassette and the direction and identity of each pair of PCR primers is as shown. The resulting combination of lysines (K) and arginines (R) for each PCR reaction is denoted by the aligned representative letters (K or R) with the Ub cassette. The restriction sites in the Ub cassette are as follows: N, *Nde*I; H, *Hpa*I; B, *Bgl*II; X, *Xba*I; Bsm, *Bsm*I; Xho, *Xho*I, S, *Sal*I; A, *Afl*II; K, *Kpn*I.




I. PCR selection after ligation for full length Ub derivatives with a single lysine

**PCR #7. Lysine 63 Ub PCR selection.**




**PCR #8. Lysine 48 Ub PCR selection..**

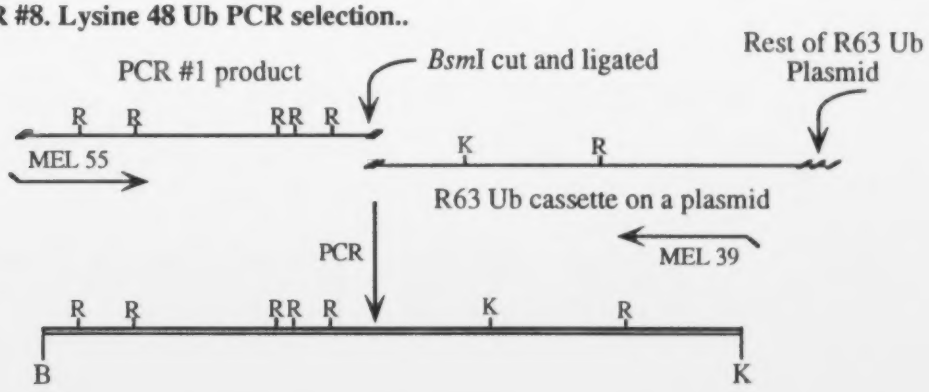
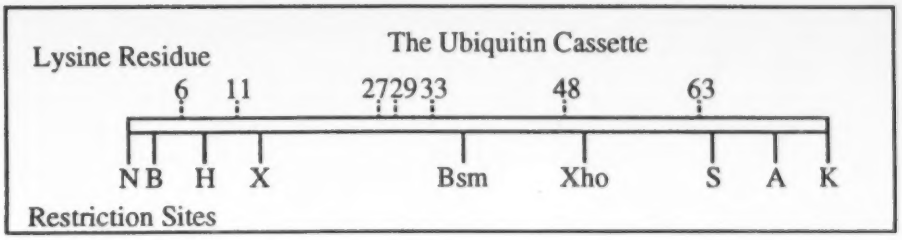


FIGURE B.5

**STEP 3 in the multistep PCR strategy designed of the construction of single lysine derivatives of Ub: Creation of R7, a UbΔ derivative with all seven lysines converted to arginines.**

The third step in the construction of this series of single lysine Ub derivatives was to create a Ub derivative lacking all seven lysine (K) residues by converting them to seven arginines (R7 Ub). The R7 derivative was made by ligating portions of previously available Ub cassettes to generate a full length Ub cassette lacking all lysines. The *BamH*I-*Xho*I fragment of the R63Ub vector previously made and described (Figure B.4) was ligated with the *Xho*I cut PCR#7 product. Successful ligations were selected by using a portion of the ligation reaction as template in the **PCR#9** reaction. The full length R7 Ub cassette was amplified using MEL55 as the 5' primer and MEL39 as the 3' primer to maintain the arginine conversion present in the cassette.

To convert this R7 Ub cassette into a UbΔ form (deleted for the C-terminal glycines residues Gly75 and Gly76), the *Bgl*II-*Sal*I fragment of PCR #9 product was used to replace a wild type Ub gene cassette lacking the C-terminal residues present on a *TRP1* vector. PCR products #9 is shown aligned with the cassette and the direction and identity of the PCR primers is as shown. The resulting combination of arginines (R) for the PCR reaction is denoted by the aligned representative letters (K or R) with the Ub cassette. The restriction sites in the Ub cassette are as follows: N, *Nde*I; H, *Hpa*I; B, *Bgl*II; X, *Xba*I; Bsm, *Bsm*I; Xho, *Xho*I, S, *Sal*I; A, *Afl*II; K, *Kpn*I.




I. PCR selection after ligation for full length Ub derivatives with a single lysine

**PCR #9. Total lysine knockout (R7) PCR selection..**

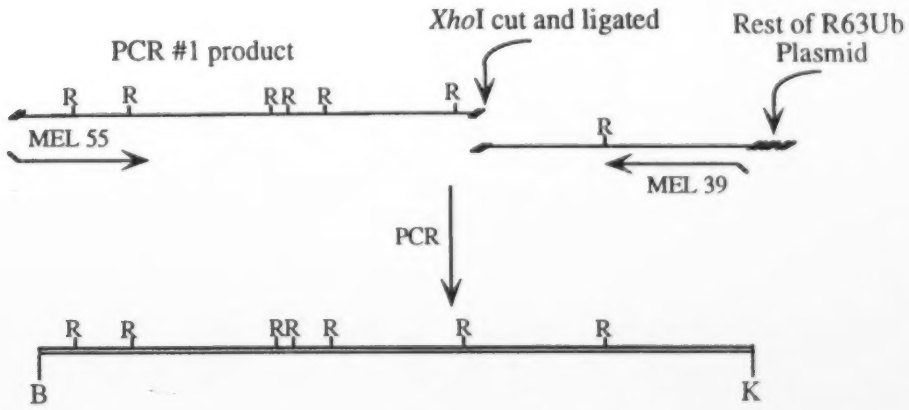


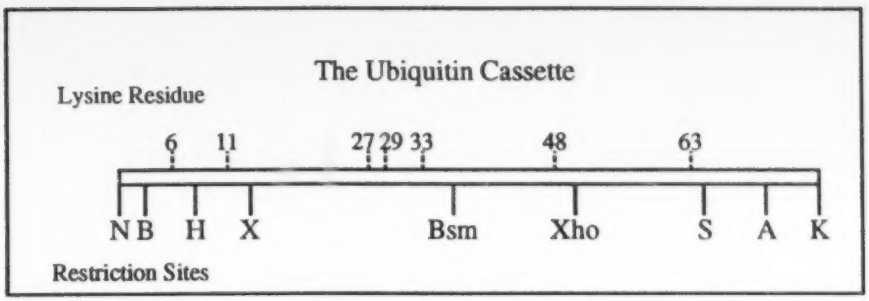
FIGURE B.6

**STEP 4 in the multistep PCR strategy designed for the construction of single lysine derivatives of Ub: Generation of UbΔ cassettes for K6, K11, K27, K29 and K33 Ub.**

A schematic diagram of the wild type ubiquitin gene cassette is shown and drawn to scale. The seven lysine (K) position are numbered and their position is shown relative to the restriction sites in the Ub cassette. The K63 and K48 UbΔ cassettes were constructed as shown in the previous figure (Figure B.5).

The final step in the construction of the series of Ub derivatives containing a single lysine at one of seven positions in Ub was to convert the six individual 112 bp PCR fragments generated in STEP 1 into full length Ub cassettes. To do this, the PCR fragments were digested with *Bsm*I and ligated to *Bsm*I digested PCR product #9 (R7 Ub). Successful ligations were selected by PCR, using primers for each PCR reaction that maintained the arginine mutations already introduced. For example, the Lysine 6 product, PCR #2a, and the PCR #9 DNA product were ligated and these sequences were then amplified with the 5' primer MEL34 and the 3' primer MEL 39. The remaining PCR products were amplified in a similar manner using the primers indicated. In all cases, the 3' primer was MEL39 which does not introduce any sequence alterations.

The full length Ub genes generated from this series of PCR amplifications were then converted to UbΔ by replacing a wild type Ub gene cassette lacking the C-terminal glycine residues (UbΔ). *Bgl*II-*Sal*I cut PCR products #2b- #6b were inserted into the *Bgl*II-*Sal*I cut UbΔ cassette present on a *TRP1* vector. PCR reactions are shown aligned with the cassette and the direction and identity of each pair of PCR primers is as shown. The restriction sites in the Ub cassette are as follows: N, *Nde*I; H, *Hpa*I; B, *Bgl*II; X, *Xba*I; Bsm, *Bsm*I; Xho, *Xho*I, S, *Sal*I; A, *Afl*II; K, *Kpn*I.




I. Generation of full length Ub cassettes with PCR#2-#6.

**PCR #2b. Lysine 6.** Use MEL34 and MEL39

**PCR #3b. Lysine 11.** Use MEL33 and MEL39

**PCR #4b. Lysine 27.** Use MEL55 and MEL39

**PCR #5b. Lysine 29.** Use MEL55 and MEL39

**PCR #6b. Lysine 33.** Use MEL55 and MEL39

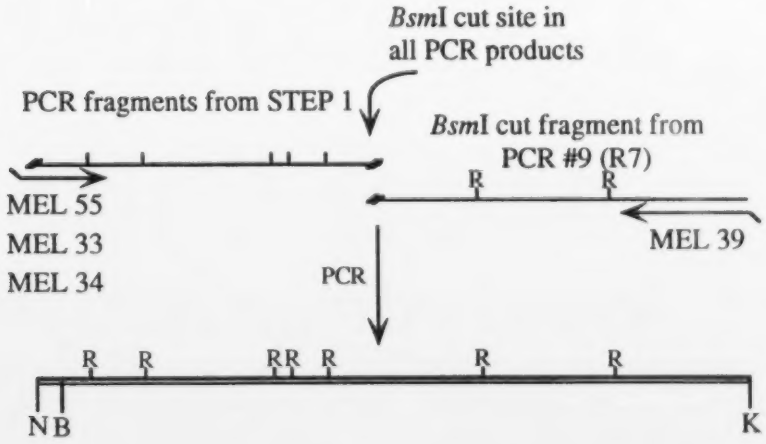
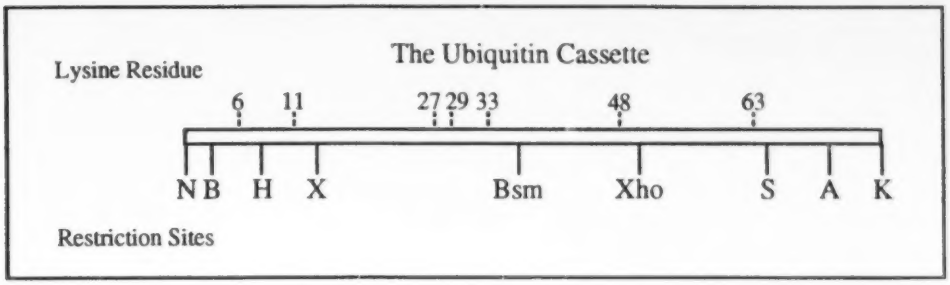


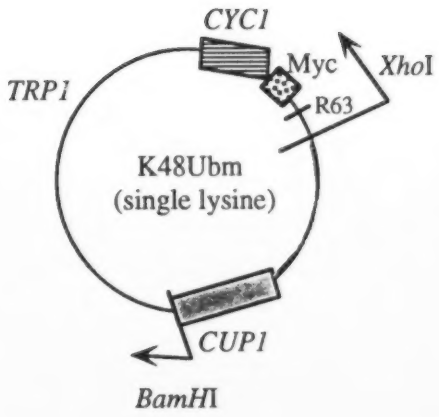
FIGURE B.7

**Construction of RRR.Ubm.**

A schematic of the wild type Ub cassette is shown for reference. Indicated are the seven lysine position and the relative position of the unique restriction sites residing between them. The cassette is drawn to scale. Also shown are schematic diagrams of previously constructed Ub derivatives present on yeast expression vectors, showing the key elements present and the restriction sites used in the construction of the RRR.Ubm derivative. The Ub cassette present on each vector is denoted by the name within the schematic. The Ub genes in all cases are 5'-3' counterclockwise. Sequences bounded by the pairs of arrows on each diagram represent the DNA fragment purified after the indicated restriction digests. **I:** Construction of RRR.Ubm. The resulting vector of this ligation is linkage deficient, due to the absence of lysine residues at the three ubiquitinateable positions 29, 48 and 63. The small *Xho*I-*BamH*I fragment from the RRK.UbΔ derivative was ligated to the large fragment generated by *Xho*I-*BamH*I digestion of K48Ubm.
Abbreviations are as follows: *CUP1*, yeast copper metallothionein promoter; *CYC1*, *CYC1* transcriptional terminator; *TRP1*, 5' P-ribosyl-anthranilate isomerase gene; Ubm, wild type Ub gene cassette with a C-terminal fusion to the sequences encoding for the myc epitope; UbΔ, wild type Ub gene cassette deleted for the final two amino acids, glycine75 and glycine76. The restriction sites in the Ub cassette are as follows: N, *Nde*I; H, *Hpa*I; B, *Bgl*II; X, *Xba*I; Bsm, *Bsm*I; Xho, *Xho*I, S, *Sal*I; A, *AflII*; K, *Kpn*I.




**I. Construction of RRR.Ubm**



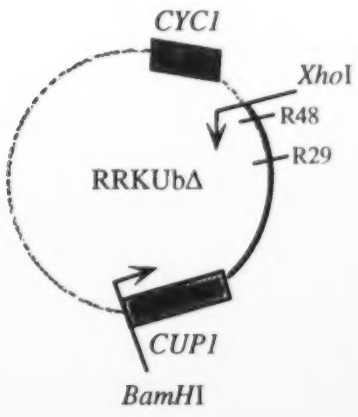
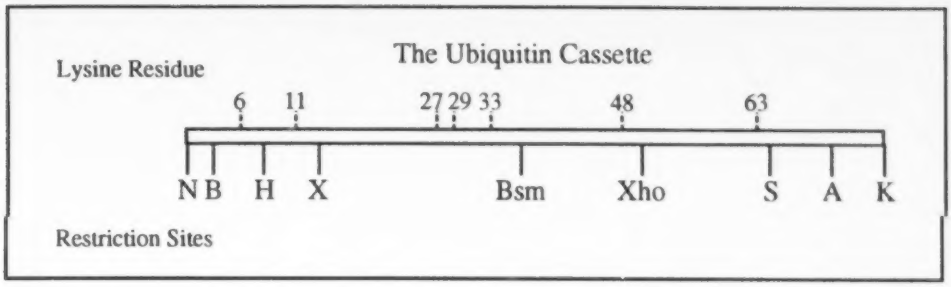



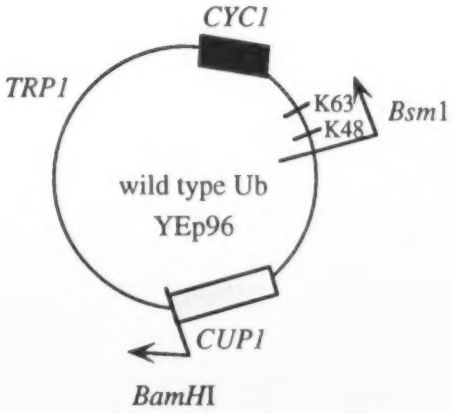
FIGURE B.8

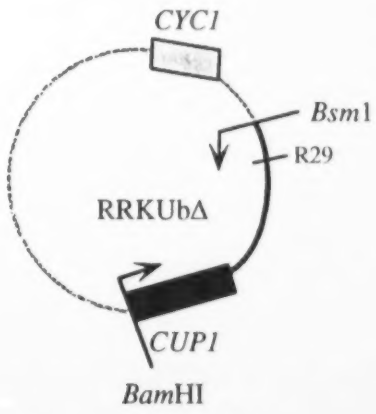
**Construction of full length Ub derivatives for phenotype analysis.**

The construction scheme used to generate full length Ub derivatives from previously constructed Ub derivatives having truncated or blocked C-termini is outlined. **I.** The R29 Ub derivative on a *TRP1* plasmid was made by exchanging portions of previously mutated Ub cassettes. The *BamH*I-*Bsm*I fragment of the RRK.UbΔ plasmid was used to replace the *BamH*I-*Bsm*I small fragment of a wild type Ub cassette. In this way, the K48 and K63 positions of wild type Ub were juxtaposed with the R29 mutation of the RRK.UbΔ cassette, resulting in an RKK Ub derivative with an intact C-terminus. **II**. The construction of the RRR, and RRK Ub derivatives with intact C-termini was done by replacing the *BamH*I-*Afl*II small fragment of a wild type Ub cassette, with the *BamH*I-*Afl*II fragment of Ubm derivatives harboring the desired mutations. The restriction sites in the Ub cassette are as follows: N, *Nde*I; H, *Hpa*I; B, *Bgl*II; X, *Xba*I; Bsm, *Bsm*I; Xho, *Xho*I, S, *Sal*I; A, *Afl*II; K, *Kpn*I.

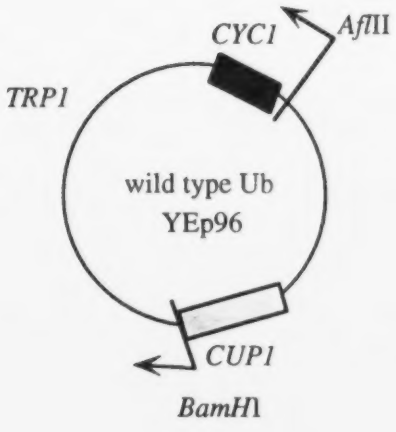



**I. Construction of R29 Ub**




**II. Conversion of RRR.Ubm, and RRK.Ubm to full length untagged Ub derivatives.**



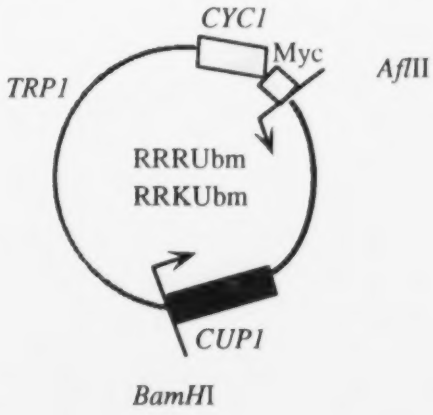
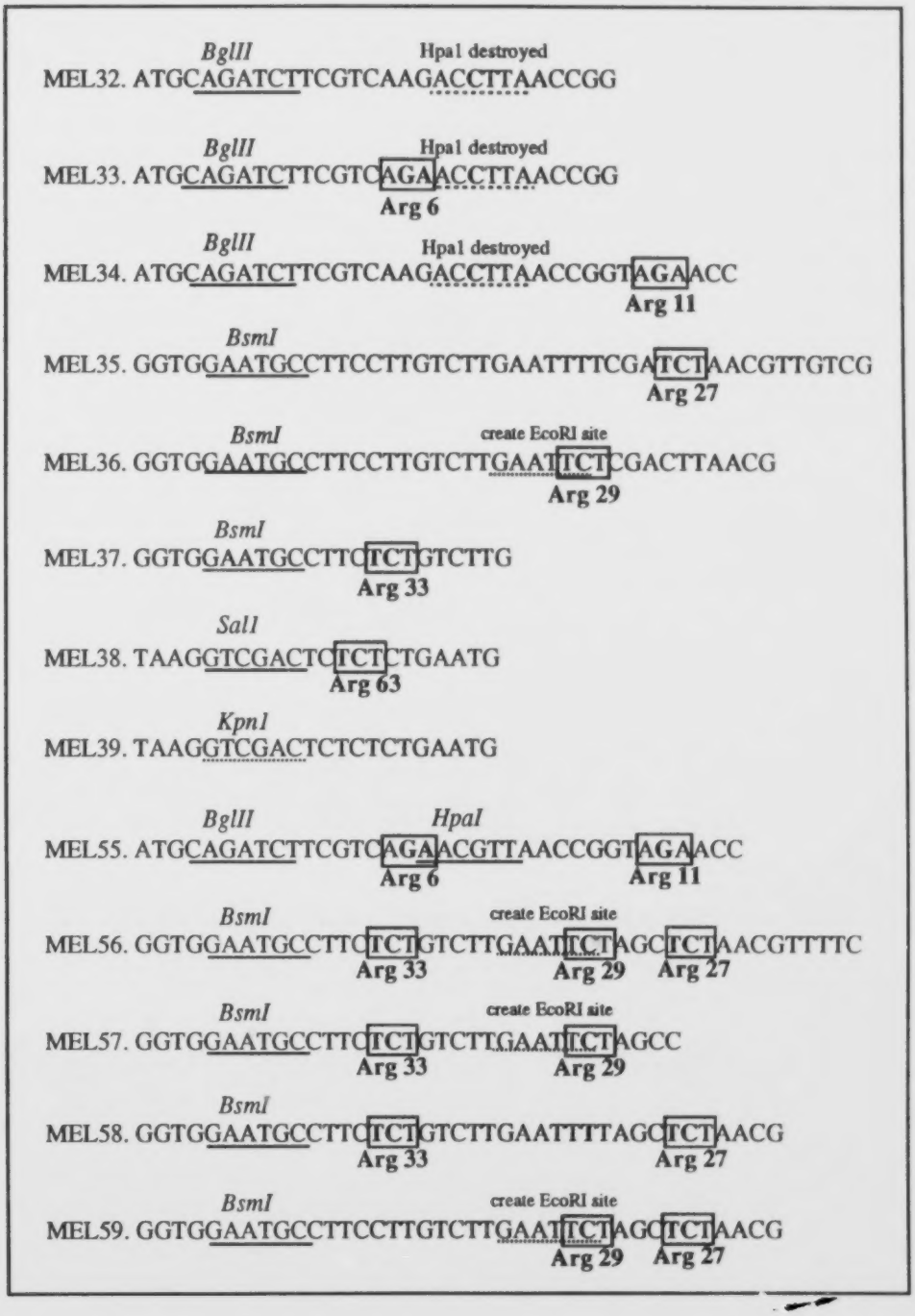



TABLE B.1

**Oligonucleotide primers used in the construction of the various Ub derivatives with lysine to arginine mutations.**

Each primer is shown 5' to 3' and the name is as referred in the text. The specific alterations introduced by the various oligonucleotides are represented by various symbols. Boxes represent the arginine codons (Arg) introduced by the primer. Numbers refer to the amino acid positions being altered. Restriction sites are of three types, denoted by an underline; ____ Restriction site present and maintained in both wild type and mutated Ub derivative. ..... .....Restriction site introduced into the Ub cassette by the oligonucleotide.....Restriction site destroyed by the oligonucleotide. Bold face bases mark the nucleotides altered from wild type by the primers used. Primers were ordered and made by the DNA Synthesis Facility at the University of Alberta, Department of Biochemistry.


BglII
Hpa1 destroyed
MEL32. ATGCAGATCTTCGTCAAGACCTTAACCGG
BglII
Hpa1 destroyed
MEL33. ATGCAGATCTTCGTCAGAACCTTAACCGG
Arg 6
BglII
Hpa1 destroyed
MEL34. ATGCAGATCTTCGTCAAGACCTTAACCGGTAGAACC
Arg 11
BsmI
MEL35. GGTGGAATGCCTTCCTTGTCTTGAATTTTCGATCTAACGTTGTCG
Arg 27
BsmI
create EcoRI site
MEL36. GGTGGAATGCCTTCCTTGTCTTGAATTCTCGACTTAACG
Arg 29
BsmI
MEL37. GGTGGAATGCCTTCTCTGTCTTG
Arg 33
SalI
MEL38. TAAGGTCGACTCTCTCTGAATG
Arg 63
KpnI
MEL39. TAAGGTCGACTCTCTCTGAATG
BglII
HpaI
MEL55. ATGCAGATCTTCGTCAGAACGTTAACCGGTAGAACC
Arg 6
Arg 11
BsmI
create EcoRI site
MEL56. GGTGGAATGCCTTCTCTGTCTTGAATTCTAGCTCTAACGTTTTC
Arg 33
Arg 29
Arg 27
BsmI
create EcoRI site
MEL57. GGTGGAATGCCTTCTCTGTCTTGAATTCTAGCC
Arg 33
Arg 29
BsmI
MEL58. GGTGGAATGCCTTCTCTGTCTTGAATTTTAGCTCTAACG
Arg 33
Arg 27
BsmI
create EcoRI site
MEL59. GGTGGAATGCCTTCCTTGTCTTGAATTCTAGCTCTAACG
Arg 29
Arg 27

## Appendix C. Large Scale Purification of Ub derivatives from *E. coli.* and potential applications.

### C.1. Introduction.

Isolated multiUb chains capable of signaling a test protein for degradation were found to be composed of numerous Ub monomers attached through the C-terminus of one Ub protein to the ε-amino group of a lysine (K) residue at position 48 (K48 multiUb chain; Chau *et al.*, 1989; Chen *et al.*, 1990). However, it has now been demonstrated that Ub-Ub conjugates can form at two lysine positions in addition to position 48 (Chapter 2). *In vivo* experiments in *S. cerevisiae* demonstrated that Ub-Ub linkages can form at positions 29, 48 and 63. Furthermore, the K63-dependent Ub-Ub linkage appeared to play an important role in the yeast stress response, but not in protein degradation (Chapter 3). A greater understanding of the requirement of the K29 and K63 alternative linkages, however, required the identification of cellular proteins targeted by these linkages.

This Appendix outlines a protocol developed for the large scale (milligram) purification of Ub derivatives from *E. coli.* The use of the purified Ub derivatives to chemically sythesize Ub dimers for monoclonal antibody production will be discussed as a method to identify proteins targeted by the alternative linakges. The linkage-specific antibodies, in turn, may allow cellular proteins to be identified which are covalently bound by multiUb chains containing the K29 and K63 Ub-Ub linkages. The identification of such proteins will enable numerous, unresolved questions to be addressed, including the effect of protein stability when ubiquitinated by the K29 linkage and the manner in which the K63 Ub linkages are utilized by RAD6 and UBC4 in DNA repair and the stress response, respectively. Furthermore, the identification of proteins targeted by K29 and K63 linkages may resolve the question of targeting specificity of the Ub-conjugating enzymes. This question asks whether the same proteins are targeted in more than one manner, or if distinct subsets of proteins recognized by Ub conjugating enzymes are targeted by different linkages in order for the Ub conjugating enzymes to carry out their intracellular functions.

### C.2. Purification of Ub derivatives.

Ub mutants were used for protein purification. These Ub derivatives had been previously constructed and carried lysine-to-arginine conversions at all but one of the seven lysine in Ub (Chapter 2). Full length Ub derivatives expressing either lysine 29 (K29), lysine 48 (K48) or lysine 63 (K63) were overexpressed from the $T_7$ promoter of a modified pET3a plasmid (Rosenberg *et al.*, 1987) in *E. coli* and purified in milligram

quantities, as outlined below. Large quantities (80 mg) of protein were isolated for each Ub derivative.

A modified pET3a plasmid was used to overexpress the three Ub derivatives in *E. coli* when the Ub genes were placed behind the T7 promoter. After induction, *E. coli* whole cell lysates contained detectable quantities of Ub. A protein band appears specifically upon induction of the T7 promoter, as shown in Figure C.1. Wild type (wt) Ub is not observed (Lane wt -) until heat induction (Lane wt +). Similarly, the vector alone does not produce the low molecular weight band regardless of the induction conditions (Lane vector, - and +). Also shown is the efficient overexpression after induction of Ub derivatives carrying a single lysine (K) at positions 29 (K29), 48 (K48) and 63 (K63).

Ub has been available in commercially pure form for some time, and different protocols are available for Ub purification (Ecker *et al.*, 1987; Beers and Callis, 1993; Jabusch and Deutsch, 1983; Wilkinson, 1988). One standard method for Ub purification from *E. coli* involves heat treating the cells (85°C) to precipitate the majority of proteins, while leaving the Ub protein in solution. This method was used as a rapid first step in the isolation of Ub from the vast majority of cellular proteins (Ecker *et al.*, 1987). However, upon using this method, it was observed that a significant portion of the overexpressed Ub protein was lost with the cellular precipitate. As shown in Figure C.2a, after heat treatment, the Ub protein is found distributed in both the protein pellet (P lane) and in the soluble supernatant (S lane). An equivalent amount of whole cell lysate (WCL lane) indicates the comparative abundance of Ub before heat treatment. A comparison to the Ub levels after heat treatment reveals that much of the Ub protein was lost in the pellet.

A Ub purification protocol was developed which did not incorporate heat treatment of induced *E. coli* lysates as a purification step. Rather, a two-column FPLC purification protocol was used, as outlined in detail in the Methods section. The first purification step used an anion exchange column (MonoQ), where pH conditions were set so that the Ub protein eluted in the flowthrough (Ft) volumes (and did not interact with the column). As shown in Figure C.2b, passage of an equivalent aliquot of cell lysate to that represented in Panel A over a MonoQ column successfully enriched the solution for the Ub protein. Specifically, the first MonoQ flowthrough fraction (Ft 1) yielded highly purified Ub protein. Because detectable Ub was also present in the second flowthrough (Ft 2) fraction, this volume was combined with the first before the subsequent purification steps. A comparison of the abundance of Ub present in Ft 1 and the original cell lysate (WCL) demonstrated the high efficiency of protein purification. Therefore, this data indicates that this procedure was not only successful at preventing the significant loss of Ub found for

the heat treatment step, but also provided a rapid, straightforward method for generating near pure samples of Ub protein.

The subsequent purification step used a Hi-Load G-75 size exclusion column. As shown in Figure C.3, this purification step yielded essentially pure Ub protein for the K29, K48 and K63 Ub derivatives. Also shown is a sample of commercially available wild type Ub. In contrast to the purified K29, K48 and K63 Ub derivatives, the commercially available Ub protein contains a detectable contaminant, indicted by an asterix (*). Therefore, the FPLC-based purification protocol used for the Ub derivatives was both efficient and practical.

### C.3. Proposed application of the purified Ub derivatives.

As mentioned above, future experiments would utilize the purified Ub derivatives to create monoclonal antibodies which specifically recognize the K29 or K63 Ub-Ub linkages. The purified monomeric Ub derivative proteins would be subjected to chemical synthesis to create Ub-Ub dimers joined through K29, K48 or K63. Chemical manipulation of purified Ub protein had been previously shown to enable milligram quantities of artificial Ub-protein conjugates to be isolated which were joined by an isopeptide bond between the C-terminus of Ub and an internal lysine residue on the test protein (Tamura *et al.*, 1991; Wilkinson, 1987). Based on this procedure, milligram quantities of Ub-Ub conjugates joined through specific lysine residues will be prepared, as the chemical synthesis is predicted to be indistinguishable from that used to generate Ub-protein conjugates.

The chemical reactions used to artificially create Ub-protein conjugates involve the chemistry of carboxylic acids and primary amines. Specifically, the reactive group of the Ub C-terminus (carboxylic acid) and the nucleophilic character of the primary amino groups of lysines have been previously used to covalently attach the C-terminus of Ub (the donor protein) to the ε-amino group of lysine residues in test acceptor proteins (Wilkinson, 1988). This method will be applied to include the lysines present on the Ub protein in order to create Ub-Ub conjugates joined by the C-terminus of one Ub moiety and the ε-amino group of the next. This Ub-Ub linkage is predicted to be indistinguishable from the natural Ub-Ub isopeptide bond.

The chemical synthesis steps for the formation of a Ub-Ub conjugate are outlined in Figure C.4. Briefly, the C-terminus of Ub can be specifically activated in a multi-step reaction. First, the enzyme, trypsin, is used to digest Ub under defined conditions to specifically cleave only the peptide bond between Arg74 and Gly75 to yield Ub lacking the two C-terminal glycine residues (UbΔ) and a free glycylglycine dipeptide (Wilkinson,

1988). In the presence of high concentrations of glycylglycine ethyl ester (an ester modification of the two glycine residues cleaved from Ub), trypsin will catalyze the formation of ethyl ester-Ub in a reversal of the cleavage reaction. This has been referred to as a transpeptidation reaction and up to 50% of the starting material can be converted to this form (Wilkinson, 1988). This Ub ethyl ester is then used as an intermediate for subsequent reaction steps. First, the ester is converted to the acyl hydrazine, and subsequently to the acyl azide, a very reactive chemical group. In the presence of a nucleophile, the acyl azide undergoes nucleophilic attack to form a Ub-conjugate. Furthermore, it has been demonstrated that the amino groups of peptides and proteins are sufficiently reactive for nucleophilic attack by the Ub acyl azide, resulting in the formation of Ub-protein conjugates.

By using a Ub derivative which has only one lysine residue available, and therefore one ε-amino group and one α-amino group (at the N-terminus) as the nucleophile for the Ub acyl azide, the resulting Ub conjugates formed will occur only at the N terminus or internal lysine residue of the protein. The conversion of the remaining lysines in each derivative to arginine will prevent them from being available for nucleophilic attack in the final conjugation reaction. Additionally, it may be possible to specifically modify the N-terminal amino group (Hershko *et al.*, 1984) to increase the yield of Ub-Ub conjugates formed specifically at the ε-amino lysine group.

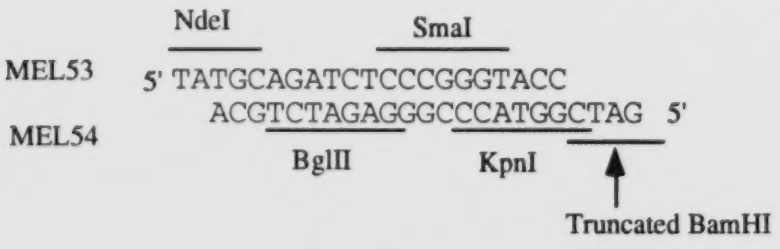
If the Ub molecule is denatured, then trypsin will cleave the Ub protein into peptide fragments (Wilkinson, 1988). Therefore, for the Ub derivatives to be useful for chemical conjugation, they must be correctly folded to ensure that cleavage is limited to the C-terminus. To test this, the purified K63 Ub derivative (as a control for K29) was reacted with trypsin and the resistance to degradation observed. As shown in Figure C.5, the K63 Ub derivative was resistant to trypsin cleavage. Wild type Ub (commercial source) remained at the same molecular weight and abundance with (+), or without (-), trypsin cleavage, suggesting, as expected, that the protein was not degraded into peptides. Moreover, the K63 Ub derivatives also appeared resistant to trypsin cleavage, as the abundance of Ub monomer with (+) and without (-) trypsin treatment was not significantly different. Therefore, the purified Ub proteins are suitable for this approach to chemically create Ub conjugates. Through the use of large quantities of purified Ub protein, sufficient amounts of the chemically synthesized Ub-Ub conjugates should be obtained for antibody generation. The generation of linkage specific antibodies is not trivial. However, after the initiation of this project, it was reported that K48-linkage specific antibodies had been created (Fujimuro *et al.*, 1994)). Therefore, it may be that this published methodology will be applicable to the generation of K29 and K63-linkage specific antibodies.

The K29 and K63 Ub conjugates have not been chemically created (see above), and therefore it has not been possible to generate antibodies specific for these linkages. When available, however, use of these antibodies should allow for great insights into the role of these alternative linkages *in vivo*. As mentioned above, it may be possible to identify protein targets that are modified by alternative ubiquitination. One way to approach this may be through immunoprecipitation. Alternatively, Western analysis of cell lysates may indicate the number and abundance of those proteins which are ubiquitinated by the alternative linkages, and perhaps lead eventually towards their identification. Additionally, it will prove interesting to observe if protein targeting by the alternative linkages is affected by the introduction of stress conditions, or cell cycle progression. Lastly, comparative Western analysis of yeast cell lysates from strains with, or lacking, specific E2 genes may reveal different ubiquitination patterns. In turn, this may prove to be a means of identifying protein targets specific for a given E2.

## Materials and Methods

**Construction of a modified pET3a *E. coli* overexpression vector.**

To introduce useful cloning sites into the pET3a overexpression vector (a gift from D. Stuart), two complementary oligonucleotides (MEL 53, MEL54) were used to introduce multiple unique restriction sites into the limited multiple cloning sites already present. The sites introduced included *Nde*I, *Bgl*II, *Sma*I, and *Kpn*I. The *BamH*I site was destroyed. The oligonucleotides were ordered from the DNA Synthesis facility, University of Alberta. The oligonucleotides were annealed by heating a 20 µl volume in TE to 55°C for 5 minutes followed by gradual cooling to room temperature. Phosphorylation of the 5' ends was carried out by mixing 5 µl of annealed MEL53 and MEL54, 4 µl 5x ligase buffer, and 2 µl T4 Kinase in a volume of 20 µl. After incubating for 1 hour at 37°C, the reaction was stopped by adding 1 µl of 0.5M EDTA. The oligos were introduced to pET3a by ligating the large *Nde*1-*BamH*I digested fragment of pET3a with the annealed oligos.



The Ub cassettes are conveniently transplanted from one vector to the next as a *Bgl*II-*Kpn*I fragment. The pET3a vector, however, had a *Bgl*II site located in a noncoding region in addition to the *Bgl*II site introduced by the oligonucleotides. To destroy the second *Bgl*II site in the pET3a vector, The *Bgl*II site was cut, filled in, and religated to generate a ClaI site. The *Bgl*II site was filled in by adding 2 µl of 2 mM dNTP mix was added in addition to 1 µl of Klenow enzyme (BRL) to the 15 µl digest of pET3a. After reacting for 1 hour, the reaction was stopped by extraction with phenol/chloroform followed by ethanol precipitation. The DNA was pelletted after incubation at -80°C and the pellet was resuspended in 10 µl of milliQ water. 3 µl of 5x ligase buffer and 2 µl of T4 DNA ligase was added to facilitate the ligation of the now blunt ends.

The K29, K48 and K63 Ub derivatives were removed from the Yeast vectors as *Bgl*II-*Kpn*I fragments and placed behind the T7 promoter of the pTer31 plasmid. Each Ub derivatives was sequenced as outlined in Chapter 2, but the sequencing primers were complementary to the T7 promoter:
(MEL100. CGAAGATTAATACGACTCDACTATAGGG) and
the T7 Terminator: (MEL101. ATTGCTCAGCGGTGGCAGCAGCCAACTCAGC).

**Ub Protein overexpression from the T7 promoter.**

Protein overexpression from the T7 promoter was carried out by heat induction using a two plasmid expression system. The kanamycin resistant pGP1-2 plasmid (a gift from D. Stuart), carrying the T7 Polymerase, is present in the -80°C competent stocks of the BL21 *E. coli* strain. The second ampicillin resistant plasmid is a pTer31 derivatives with the T7 promoter. A single, freshly transformed colony from a kanamycin/ampicillin plate was inoculated into 5 ml of LB (1 liter: 10 g bactotryptone, 5 g yeast extract, 5 g NaCl) with ampicillin and kanamycin and grown overnight at 30°C with shaking. The next morning, the culture was diluted 1:40 into fresh LB plus Amp/Kan (approximate $O.D._{590}$= 0.1) and grown at 30°C until the optical density reached 0.3. The cells are then induced at 42°C for 1 hour, followed by continued growth at 37°C for 1 hour. For small volumes, test tubes were transferred to a 42°C water bath for induction, and for larger volumes, the flasks were heated rapidly under the hot water tap and the temperature monitored using a thermometer wiped in 95% ethanol. Cells are then pelletted by centrifugation, and either frozen at -80°C for further protein purification, or lysed directly in 2x SDS PAGE sample buffer.

**Large Scale Ubiquitin Purification from *E. coli*.**

*a. Cell harvest and Lysis.*

Ubiquitin derivatives were overexpressed in *E. coli* using the standard overexpression protocol for the T7 promoter in 2 liter volumes. Cells from these cultures were pelletted in 1 liter bottles in a Damon IEC centrifuge (3,000 x g, 20 min) The cells were combined in a total volume of 15 ml of lysis buffer (5 mM Tris pH 7.5, 1 mM EDTA, 1:100 dilution of standard protease inhibitors including 2.5 mg/ml chymostatin, 5 mg/ml leupeptin, 2.5 mg/ml pepstatin, 5 mg/ml antipain and 5 mg/ml aprotinin) and at this point were stored at -80°C without harm. After 4 liters had been induced (30 ml total volume of resuspended cells) the cells were lysed by a French Pressure Cell (AminCo, 40,000 psi maximum) at 20,000 psi in a single pass. The cellular debris was then pelleted (40,000 K, 1 hr, 4°C, in a Ti70 rotor). The clarified supernatant can be frozen at -80C without harm at this point.

b. Purification of Ub by FPLC.

Ubiquitin can be purified in a two column process using an FPLC with a MonoQ High-Load column followed by a Hi-Load Sephadex G-75 size exclusion column. The MonoQ column was equilibrated with 5 mM Tris pH 7.5 (filtered). The 30 ml of clarified lysate was further clarified after centrifugation by passage through a 0.22 µm filter before loading onto the column. This large volume was loaded using the Superloop (50 ml capacity). Ubiquitin eluted from the column in the first and second flowthrough fractions (50 ml) without an increase in salt concentration. Standard protease inhibitors were added to the flowthrough fraction, and the volume was reduced by lyophilization. For lyophilization, the protein sample is frozen in a thin film on the inside of a 600 ml lyophilization flask, and dried overnight. The protein was then resolubilized in 10 ml of milliQ water, clarified by filtration, and loaded onto a high load G-75 column (equilibrated in 50 mM Tris pH 7.5) in 5 ml aliquots. This column was run at the maximum flow rate of 1 ml per minute. Ubiquitin eluted as a single peak (at 87 minutes) in a 15 ml volume. This Ub fraction was then lyophilized and resuspended in a highly concentrated solution, or dialyzed first against milliQ water followed by lyophilization. Protein concentration of the purified Ub was determined by spectrophotometry. Specifically, the 1 mg/ml extinction coefficient for pure Ub is an optical density of 0.16 at 280 nm (Wilkinson, 1988). Up to 8 mg of purified Ub derivative protein per liter was obtained using this method.

**Trypsin digestion of Ub protein.**

500 µg of Ub protein, from a commercials source (Sigma) or from the K63 Ub protein purified as above, were either mock treated or treated with trypsin. The 500 µg of

protein was lyophilized in eppendorf tubes, and to this was added 10 µl of 50 mM Hepes pH 7.0 to resuspend the protein. Both wild type and K63 Ub samples were done in duplicate. To one tube of each, 1.0 µl of a fresh trypsin stock solution (50 mg/ml in 10 mM HCl) was added, and to the other, 1.0 µl of 10 mM HCl was added (mock treated). After the tubes were mixed, the four samples were incubated at 37°C overnight (22 hr). The next day, 11 µl of 2 x SDS loading buffer was mixed and the entire sample was run on an 18% acrylamide gel.

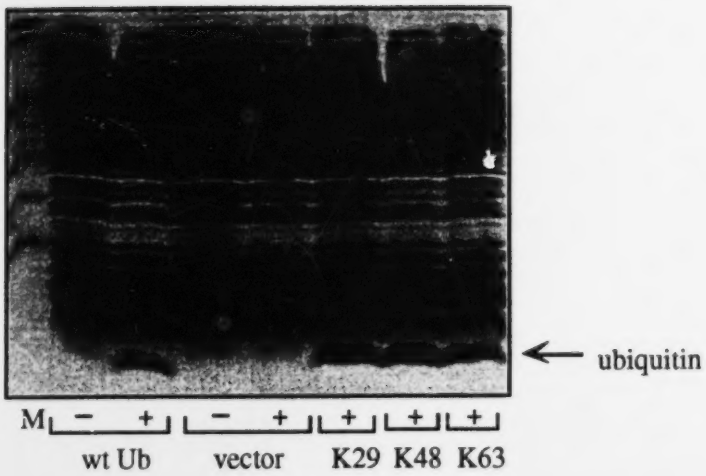



FIGURE C.1

**Ub and Ub derivatives are overexpressed from the T7 promoter in *E. coli.***

Shown is a Coomassie Blue stained 18% acrylamide gel of *E. coli* whole cells lysates before (- lanes ) and after (+ lanes) overexpression of Ub and Ub derivatives from the T7 promoter. A modified pET3a vector with wild type (wt) Ub, no gene (vector only), or one of three Ub derivatives with all lysines (K) converted to arginines excepting one residue at position 29, (K29), or position 48 (K48) or position 63 (K63) were transformed into the BL21 *E. coli* strain with the pGP1-2 T7 Polymerase-encoding plasmid. Overexpression was induced by heat treatment (see Methods). The position of the overexpressed Ub protein is as indicated. The M lane contains commercial low-range molecular weight markers, of decreasing molecular weight from top to bottom, of 110 kDa, 80, 50, 33, 28, and 19 kDa.

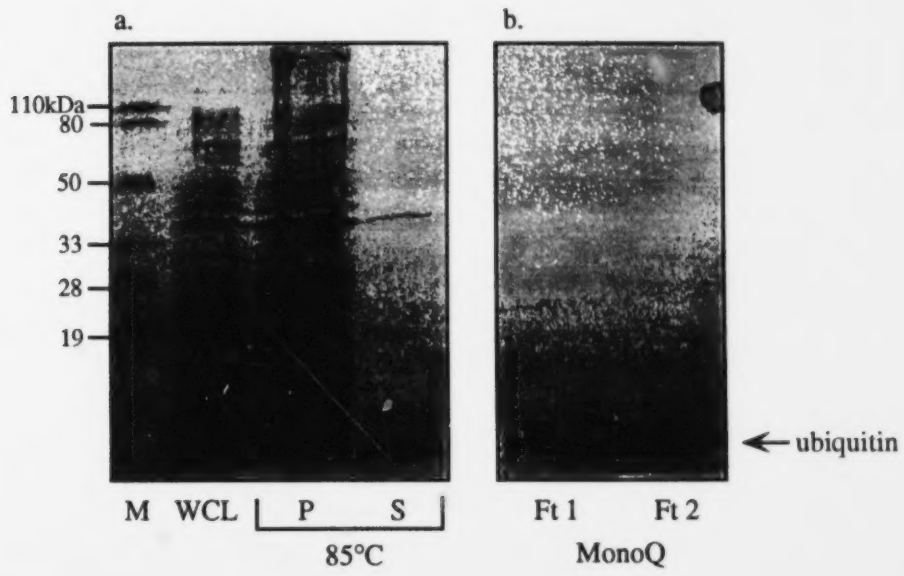



FIGURE C.2

**FPLC Purification (MonoQ) of Ub from *E. coli* maximizes yield.**

Shown are Coomassie stained, 18% acrylamide gels. Each lane represents an equal aliquot of original *E. coli* whole cell lysate after Ub overexpression from a T7 promoter. This experiment was done to compare the yield of Ub protein after different purification procedures. **Panel a.** Equal volumes of *E. coli* whole cell lysate before (WCL lane) and after (S and P lane) heat treatment at 85°C. The heat treated sample was divided into the soluble (S) and insoluble (P, pellet) fractions. **Panel b.** An equal volume of whole cell lysate to that in Panel a (before heat treatment) was run through a MonoQ High Load Sephadex column (see Methods), and the first (Ft 1) and second (Ft 2) flowthrough fractions were collected and concentrated. The position of Ub protein is as indicated. M lane contains commercial low-range molecular weight markers as in Figure C.1.

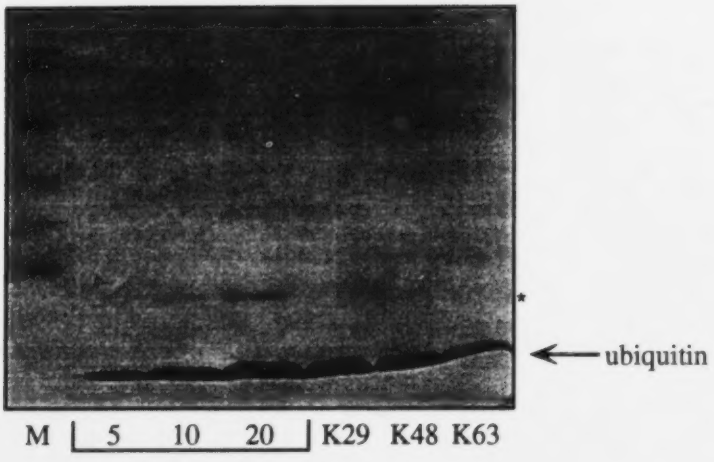



FIGURE C.3

**Large Scale purification of Ub derivatives from *E. coli* using a two column FPLC purification scheme.**

Shown is a Coomassie stained 18% acrylamide gel with both commercially available Ub protein (wild type Ub, Sigma) and three Ub derivatives purified as outlined in the Methods section. 5 μg, 10 μg and 20 μg samples of the Sigma Ub was used, and the contaminant present in the higher concentrations is indicated by the asterix (*). Approximately 10 μg of each of the purified Ub derivatives, from the 80 mg of purified protein for each, was also included. These Ub derivatives had lysines to arginine mutations at all positions but one, at either position 29 (K29), position 48 (K48) or position 63 (K63). The M lane contains commercially available low-range molecular weight markers, as in Figure C.1.

FIGURE C.4

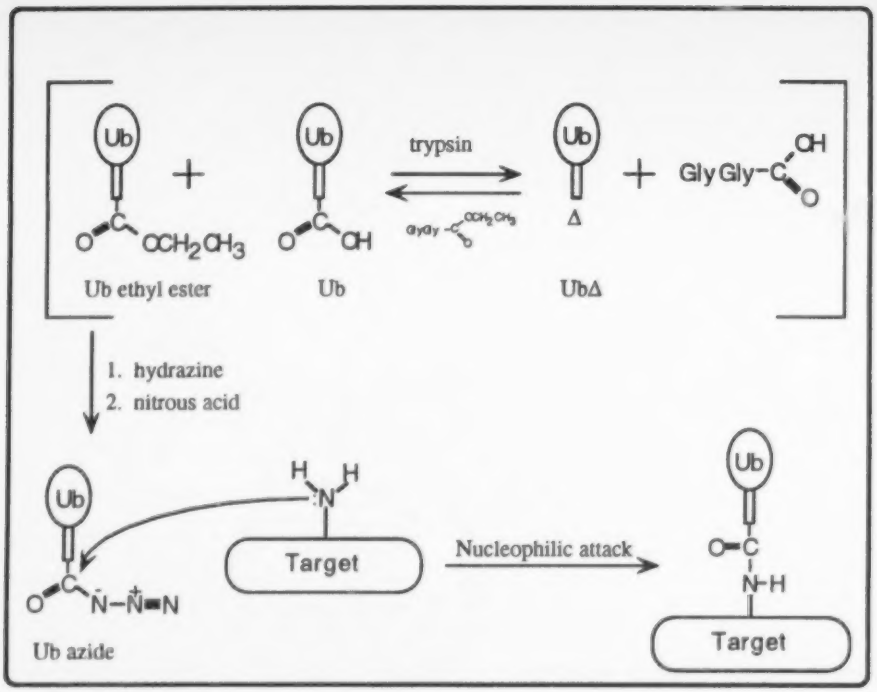
**The chemical synthesis steps for the creation of Ub-protein conjugates.**

Shown is a schematic detailing the (I) chemical synthesis steps previously used to create Ub-protein conjugates, and (II) the proposed synthesis of Ub-Ub conjugates linked through positions K29 or K63 using the same chemical steps.

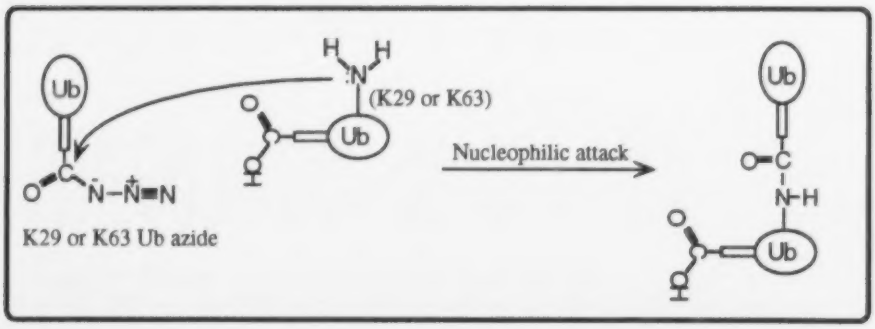
**I.** Within the square brackets is shown the forced, reversible tryptic cleavage of the C-terminal Gly75 Gly76 amino acids from wild type Ub in the presence of glycylglycine ethyl ester. The results is the formation of both UbΔ, and Ub ethyl ester. Subsequent treatment of isolated Ub ethyl ester generates the reactive Ub-azide derivative, which is capable of forming a covalent bond with primary amino groups. The result is an isopeptide bond between the C-terminus of Ub and the ε-amino group of the protein substrate.

**II.** The Ub-azide of a single lysine derivative is reacted with an un-derivatized single lysine Ub derivative, to potentially yield a Ub-Ub conjugate through as defined lysine residue. It is also feasible that Ub-Ub conjugates may be generated which contain three or more Ub moieties using this procedure to create multiUb chains of homogenous alternative Ub-Ub linkages.

I Ub-protein conjugate




II Ub-Ub conjugate (K29 or K63)



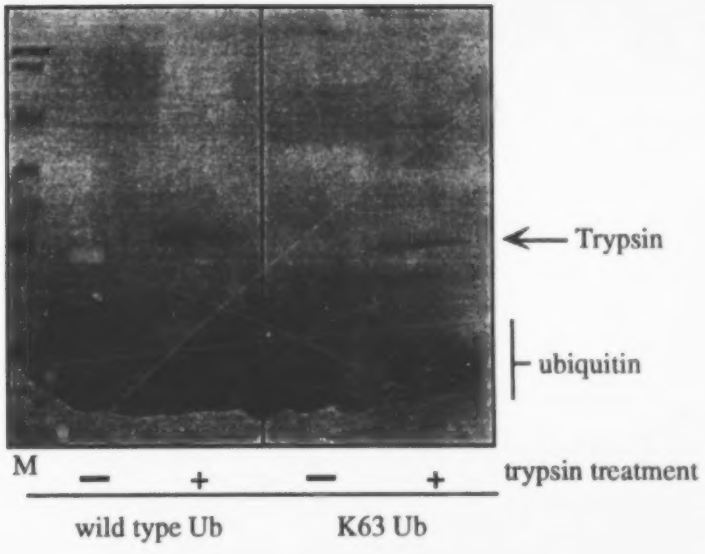



FIGURE C.5

**A purified Ub derivative is resistant to trypsin cleavage.**

Shown is a Coomassie stained 18% acrylamide gel. Samples include both commercially purified wild type Ub (Sigma) and a purified K63 Ub derivative before (- lanes) and after (+ lanes) trypsin cleavage (see Methods). 500 μg of Ub protein was used in each experiment. The large amount of Ub used in the experiment lead to a distortion of the Ub protein bands, as shown. A unique protein band appearing after trypsin cleavage was assumed to be trypsin itself, as indicated. M lane contains low range molecular weight standards as described in Figure C.1.

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